THE POPULATION GENETIC STRUCTURE OF *Quadrula aurea* (BIVALVIA: UNIONIDAE), A THREATENED FRESHWATER MUSSEL IN CENTRAL TEXAS

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The anthropogenic alteration of riverine ecosystems has led to declines in the abundance and diversity of freshwater mussels (Bivalvia: Unionoida) worldwide. Central Texas is home to a diverse freshwater mussel fauna including three candidates for federal listing under the Endangered Species Act. Surveys conducted over the last few decades suggest many of the endemic freshwater mussel species in Texas exist in small isolated populations that may be vulnerable to the deleterious effects of genetic diversity loss. Microsatellite primers from two closely related species were used to identify a set of genetic markers that functioned in the golden orb (*Quadrula aurea*). Microsatellite markers were then applied to document the population genetic structure of *Q. aurea* within and among three connected river drainages in southeastern Texas. Gene flow within existing *Q. aurea* populations appears high indicating little potential for genetic issues stemming from isolation and inbreeding. Two weakly divergent admixed populations were identified occupying the San Antonio and Guadalupe/San Marcos rivers. Population genetic structure was related to river basin affiliation, but results for environmental factors were unresolved. Current effective population size estimates are large for the Guadalupe/San Marcos drainage and moderately large for the San Antonio drainage and there is no clear genetic evidence of contemporary population declines. Transport in the glochidial phase by a highly mobile host fish, the channel catfish (*Ictalurus punctatus*), may provide a mechanism for maintaining connectivity among spatially discrete mussel beds and deserves further study. Information on the occurrence and habitat associations of *Q. aurea* and

two other threatened freshwater mussel species was documented. Quantification of the population genetic structure for *Q. aurea* provides important information needed for the management of this species, a baseline for understanding future changes, and insight into the factors that shape the population genetic structure of other threatened unionids in Texas.

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CHAPTER 1

INTRODUCTION

1.1 Statement of Problem

Widespread anthropogenic alteration of riverine ecosystems over the last two centuries has resulted in severe declines in the diversity and abundance of pearly freshwater mussels (Bivalvia: Unionoida; Bogan, 1993; Layzer et al., 1993; Neves et al., 1997; Gangloff et al., 2009; Haag, 2009a). Freshwater mussel species recognized as threatened in Texas appear to exist in relatively small isolated populations (USFWS 2011) where the negative effects of reduced genetic diversity may make them more susceptible to extinction (Saccheri et al. 1998; Spielman et al. 2004; Frankham 2010). However, no population genetic studies have been performed on any of Texas' freshwater mussel species and there is a lack of knowledge concerning existing genetic diversity and population genetic structure. This research seeks to develop an understanding of the neutral genetic diversity and population genetic structure of one threatened freshwater mussel species endemic to Texas, the Golden Orb (*Quadrula aurea*; Figure 1.1), in the lower Guadalupe/San Marcos and San Antonio rivers.

The lack of population genetic information is due, in part, to a lack of genetic markers with sufficient resolution to investigate genetic differentiation at the population level. Developing molecular markers *de novo* with sufficient variability to identify fine scale genetic variation can be expensive and time consuming (Abdelkrim et al. 2009). Fortunately, markers that were developed for one species are often effective in other closely related species (Krupa et al. 2002; Williamson et al. 2002; Tonnis 2006). The use of previously identified markers, when available in a closely related species, offers the potential to eliminate the costs of

microsatellite development and facilitate much needed studies of population-level subdivision and genetic diversity. The present research makes use of molecular primers originally tested in other congeneric species to identify molecular markers in *Q. aurea* with sufficient variability to be effective at the population level.

Figure 1.1. *Quadrula aurea*, with siphons exposed, located in the San Marcos River near Gonzales, Texas.

Small population size, irrespective of its influence on genetic diversity, can make a species more vulnerable to extinction from random events (Lande 1988, Lande et al. 2003, Jeppsson and Forslund 2012, Wootten and Pfister 2013). Few survey efforts have attempted to quantify population size in *Q. aurea* mussel beds (Howells 1997) and the level of relatedness between individual mussel beds is unknown - do they constitute individual populations or subsets of a larger breeding population? Perhaps more importantly, quantifiable data concerning historic vs contemporary population size/structure is nonexistent. The lack of

historic data on population size limits the ability to 1) determine if *Q. aurea* populations have declined only on a local scale or basin-wide, 2) pinpoint when *Q. aurea* populations began to decline, and 3) correlate the timing of specific natural or anthropogenic environmental changes with population change. This research applies a Bayesian coalescence-based technique in an attempt to quantify both historic and current effective population size in *Q. aurea* as well as determine the time period when population size began to change.

Central Texas is home to a diverse freshwater mussel fauna including three candidates for federal listing under the ESA - Texas Fatmucket (*Lampsilis bracteata*), Texas Pimpleback (*Quadrula petrina*), and Golden Orb (*Quadrula aurea*) - and one species, False Spike (*Fusconaia mitchelli*), that is waiting status review. The requirements for listing a species under the ESA involve a comprehensive review of the species' biology, demographic status, habitat requirements, and the factors threatening its continued existence (USFWS 2011). Most freshwater mussels are of limited economic value and have received little scientific or regulatory attention. As a result, the information required for listing is limited for many rare mussel species in Texas (Howells et al. 1996; Howells 2010). This research provides information on the demographics of *Q. aurea* as well as the occurrence and distribution of other rare unionids to help facilitate the listing process.

1.2 Objectives and Hypotheses

1.2.1 Objectives

1. Collect tissue for the extraction of DNA from Golden Orb (*Quadrula aurea*) mussel beds in the lower sections of the Guadalupe/San Marcos and San Antonio rivers in southeast Texas.

2. Utilize *Q. aurea* DNA and microsatellite primers developed for closely related species to identify a set of microsatellite markers that 1) are effective in *Q. aurea* and 2) provide sufficient variability to investigate population genetic structure.

3. Utilize microsatellite markers and genetic analysis techniques to determine the level of neutral genetic diversity and describe the population genetic structure of *Q. aurea* in the lower Guadalupe/San Marcos and San Antonio rivers.

4. Evaluate the influence of spatial structure and environmental variation on the population genetic structure of *Q. aurea*.

5. Investigate the current effective population size of *Q. aurea* and attempt to determine the magnitude, timing, and direction of changes in effective population size.

6. Use survey information to investigate the occurrence and distribution of threatened freshwater mussels in the lower Guadalupe, San Marcos, and San Antonio rivers

7. Investigate the size/age class distribution of Q. aurea in the lower Guadalupe, San Marcos, and San Antonio rivers.

1.2.2 Null Hypotheses

1 Genetic diversity does not differ among *Q. aurea* mussel beds/populations.

2 Genetic variation among *Q. aurea* populations in the lower Guadalupe, San Marcos, and San Antonio rivers is not structured spatially (i.e., population genetic structure is panmictic).

3 Environmental variation does not influence Q. aurea genetic structure.

4 The current effective population size of *Q. aurea* is not different from historic levels.

5 Size/age class distribution does not differ among rivers.

1.3 Scope

This study was designed to investigate the population genetic structure of *Quadrula aurea* in the lower reaches of the Guadalupe, San Marcos, and San Antonio rivers. The assessment was restricted to the specified region because survey work in the last few decades indicate this is the region of highest abundance for *Q. aurea* and other Central Texas threatened freshwater mussel species. DNA from a single mussel bed in the San Antonio River was used to test microsatellite primers. Mussel surveys spanned four years (2012 – 2015), but all genetic sampling occurring in 2012 or 2013.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Distribution and Conservation Status of Freshwater Bivalves

North America, with an estimated 59 genera and 302 recognized species (Bogan 2008), supports the greatest diversity of freshwater mussels (Mollusca: Bivalvia) on Earth. This diversity is driven primarily by the radiation of the family Unionidae in the lotic ecosystems of the southeastern United States where an estimated 42 genera and 271 species can be found (Bogan 2008). The state of Texas, which sits on the western edge of this important zone of unionid biodiversity, encompasses a biological crossroads where several major ecological regions overlap. As a result, Texas offers a rich diversity of aquatic habitats at the edge of an already diverse freshwater mussel assemblage. Although some debate exists on the taxonomic status of certain taxa (Burlakova et al. 2012; Campbell and Lydeard 2012), Texas is home to at least 51 recognized unionid species (Howells et al.1996) 14 of which are state or regional endemics (Burlakova et al. 2011).

Freshwater provides critical ecological services to humans therefore many of our freshwater ecosystems have been substantially altered for human benefits. Broadly speaking, anthropogenic influences on freshwater ecosystems include alterations to the timing and quantity of flow, water pollution, habitat degradation, the introduction of invasive species, and over-exploitation (Dudgeon et al. 2006; Geist 2011). These alterations have had a negative effect on mussel abundance and diversity and freshwater mussels now rank as one of the most endangered faunal groups in the North America (Bogan, 1993, Strayer 2006; Haag 2009a). Almost 70% of the species known to occur in the United States and Canada are considered

threatened to some degree and approximately 13% may already be extinct (Williams et al. 1993, Master et al. 2000). Texas waters have also been heavily impacted by anthropogenic alterations (Dahm 2005) and 15 Texas freshwater mussel species, including all 14 endemics, are now considered threatened within the state. Six state threatened species were recently elevated to candidates for listing under the Federal Endangered Species Act (USFWS 2011) and another four are currently under status review (USFWS 2009).

Freshwater mussels exist as sessile infaunal organisms that possess a set of life history attributes - relatively long lifespans, slow growth rates, low reproductive rates, and poor dispersal abilities (Bogan 1993) - that make them particularly vulnerable to rapid environmental change. The range of movement of adult mussels is limited in relation to the scale of most anthropogenic disturbances (Balfour and Smock 1995), so mussels have little capacity to seek refuge from habitat alteration. Mussels in the family Unionidae, the most abundant group of freshwater mussels in North America, may be especially sensitive to habitat fragmentation because of their unique reproductive strategy, which includes a parasitic larval stage (glochidium) that must attach to a fish host to complete development. Host specificity varies among unionids, but can be quite high (Barnhart et al. 2008) and habitat alterations that eliminate or restrict the movement of host fish can essentially "strand" unionid populations without the ability to complete their reproductive cycle (Watters 1996; Layzer and Scott 2006). Moreover, because of their infaunal nature, unionid dispersal occurs primarily in the glochidia phase and restricting the movement of host fish greatly reduces unionid dispersal capacity (Vaughn and Taylor 2000). Restricted dispersal can be especially detrimental to threatened species because of the potential to eliminate gene flow among populations (Campbell-Grant et

al. 2007), alter population-level genetic structure, and reduce genetic diversity (Junker et al. 2012; Sterling et al. 2012).

2.2 Importance of Genetic Diversity

Modern conservation approaches recognize biodiversity on three levels of organization – genes, species, and ecosystems – and recommend the development of conservation strategies for all three levels (McNeely et al. 1990). The loss of genetic diversity can compromise reproductive fitness and increase the risk of extinction through inbreeding depression and the accumulation of deleterious mutations (Saccheri et al. 1998; Spielman et al. 2004; Fankham 2010). Reduced genetic diversity is especially relevant for small isolated populations that are vulnerable to destructive feedback mechanisms between population size, inbreeding depression, and demographic and environmental stochasticity (Gilpin and Soule 1986; Saccheri et al. 1998; Fagan and Holmes 2006). A cascade of factors contributing to population extinction, referred to as an extinction vortex by Gilpin and Soule (1986), can occur when the loss of genetic variability in a small population leads to inbreeding depression, reduced fitness, lower reproductive success, and higher mortality rates. Depressed reproductive success and increased mortality rates lead, in turn, to an even smaller population with less genetic variability, more inbreeding depression, and greater vulnerability to extinction from stochastic factors.

The ability to adapt to a changing environment and thus the long-term viability of a species can be eroded through the loss of the evolutionary potential inherent in genetic diversity (Lande 1995; Frankham 2005; Barrett and Schluter 2007). Broad scale conservation

challenges such as climate change allow few options other than range displacement or trait evolution for species to cope and avoid extinction (Duputié et al. 2012). It may be especially important for species with a limited dispersal capacity, such as freshwater mussels, to maintain sufficient genetic diversity for trait evolution in the face of a changing environment.

Captive breeding programs are often utilized within the broader effort to preserve highly endangered species. Understanding genetic diversity is vital to conservation strategies that incorporate a captive breeding program. Captive populations can be subject to the loss of genetic diversity through genetic drift, inbreeding depression, and adaptation to captive conditions (Lacy et al. 1993; Ivy and Lacy 2012). Knowledge of the genetic structure of founder populations is important for developing a breeding strategy that reduces the potential for genetic problems. Furthermore, both the reintroduction of captive bred individuals and relocation efforts designed to reduce the risk of extinction from stochastic environmental processes must consider genetic differences between populations. The introduction of nonlocal alleles into a population may result in outbreeding depression and a loss of fitness (Templeton et al. 1986, Tymchuk et al. 2007). Outbreeding depression can occur because of two mechanisms. First, heterogeneous environmental factors across the range of a species result in divergent selective forces and local adaptation (Herrel et al. 2011). The genetic combination of populations that differ in terms of local adaptations can create intermediate phenotypes less suited to local environmental conditions (Hatfield and Schluter 1999, Tymchuk et al. 2007). Second, recombination and segregating during meiosis in the F1 generation may cause later generations to suffer reduced fitness due to the loss of co-adapted gene complexes and the disruption of positive epistatic interactions (Tempelton et al. 1986; Huff et al. 2011).

Ultimately, the genetic factors associated with local adaptation and co-adaptation are important to conservation efforts because they can be used to identifying evolutionarily significant units (ESU) if they exist. ESUs are variously defined (Waples 1991; Moritz 1994) but, in general, can be thought of as populations that are reproductively isolated and exhibit significant adaptive variation (Crandall et al. 2000). In short, ESUs represent segments of a population that are diverging evolutionarily and may warrant separate treatment in conservation planning.

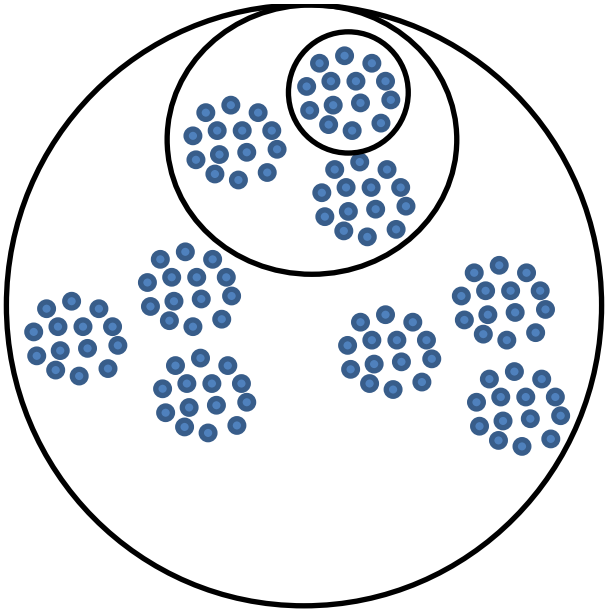

Figure 2.1. The total genetic diversity found in this example species can be partitioned across three hierarchical levels: (A) among individuals of the same population, (B) among populations, and (C) among groups of populations.

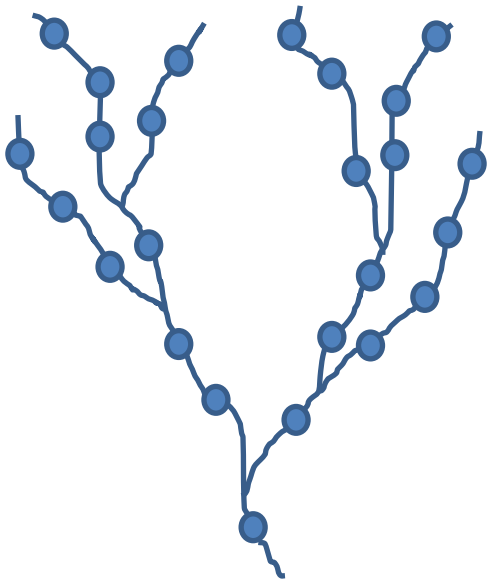

Figure 2.2. The dendritic structure of stream networks forces a two dimensional spatial relationship between pairs of populations (provided the species in question have no terrestrial phase); one population can be situated only upstream or downstream from another.

2.3 Genetic Structure, Spatial Structure, and River Fragmentation

The genetic diversity of a spatially structured population can be partitioned

hierarchically into intra-population and inter-population diversity (Nei 1973). Intra-population diversity reflects the genetic variability among individuals within populations while inter-population diversity describes genetic variation among populations and higher order groups of populations (Figure 2.1).

The amount of neutral genetic diversity within a species and how that diversity is partitioned is largely the result of opposing forces of genetic drift and gene flow (Hutchison and Templeton 1999). Gene flow between any two populations is, in turn, dependent on the degree of connectivity between populations (Keller and Largiadèr 2003; Epps et al. 2005; Hedrick 2005). Connectivity and the movement of individuals or propagules between populations promotes gene flow, maintains rates of heterozygosity, and increases the proportion of total genetic diversity attributable to within population differences. Alternatively, the loss of connectivity reduces gene flow, promotes the loss of within-population diversity through genetic drift, and increases the proportion of total diversity attributable to differences among populations. When populations are small the effects of genetic drift are strong relative to factors that promote genetic diversity (i.e. mutation), the loss of connectivity can lead to a substantial reduction in heterozygosity and a decrease in overall genetic diversity (Freeland et al. 2011).

Habitat spatial geometry is known to interact with species' dispersal ability to influence population connectivity, the dynamics of gene flow, and the partitioning of genetic diversity (Johnson et al. 1992; Campbell Grant et al. 2007; Hughes 2007; Alp et al. 2012). Riverine habitats take the form of dendritic networks, which have a hierarchically bifurcating geometry (Figure 2.2) that possess ecological properties that can restrict dispersal and affect connectivity

and genetic structure. For example, riverine species that lack a terrestrial phase are constrained to the river channel and dispersal is essentially one dimensional (i.e. up or down stream). As a result, connectivity between sets of populations is defined by dispersal along the channel network and population connectivity for species with relatively poor dispersal abilities is expected to be highest between populations adjacent in the network. Genetic structure under these conditions may reflect a linear stepping stone migration model (Kimura and Weiss 1964) and display isolation by distance (IBD) where genetic similarity decreases with increasing Euclidian distance between paired populations. Research has documented IBD structure in multiple aquatic species including insects (Westram et al. 2013), fish (Beneteau et al. 2009; Lamphere and Blum 2012), and freshwater mussels (Berg et al. 2007). In contrast, the constrained nature of movement in a riverine ecosystem may work to increase connectivity among populations of highly vagile species, provided dispersal is sufficient, by channeling movement along the network and forcing dispersing individuals to interact with multiple populations (Campbell Grant et al. 2007; Morrissey and de Kerckhove 2009). Riverine species with strong dispersal ability often exhibit panmixia where genetic structure is effectively homogeneous across populations and the majority of genetic diversity resides at the intra-population level. However, even species with relatively little genetic diversity among populations can exhibit IBD over large distances (Berg et al. 1998; Elderkin et al. 2007).

The hierarchical arraignment of dendritic networks is generally the result of elevation change. Consequently, dispersal and gene flow may be asymmetric and biased in the downstream direction due to the force of gravity (Morrissey and de Kerckhove 2009). In the case of lotic ecosystems, flowing water tends to carry gametes, larvae, and dispersing adults of

many species (Bilton et al. 2001) down-network where they may interact with downstream populations. Under conditions of asymmetric gene flow headwater populations may become isolated, producing immigrants, but receiving few emigrants, and may diverge genetically due to drift (Morrissey and de Kerckhove 2009). Genetic diversity in down-network populations, on the other hand, may be maintained by the receipt of immigrants from headwater populations with differing allele frequencies. The landscape scale affect is a general increase in genetic diversity from upstream to downstream populations (Alp et al. 2012, Lamphere and Blum 2012).

The fragmentation of the longitudinal river corridor by weirs, dams, hydropower facilities and culverts represents a major global human impact on running waters (Jungwirth 1998). Natural patterns of gene flow based on spatial geometry and dispersal ability can be substantially altered by barriers that fragment lotic habitats (Horreo et al.2011). Manmade barriers such as dams can block movement in one or both directions with differing consequences for genetic structure. Small barriers may only limit dispersal in the upstream direction and either enhance the effects of asymmetric gene flow (Junker et al. 2012) or create an asymmetric pattern where one would not naturally occur. Larger barriers that limit movement in both directions may effectively eliminate population exchange and accelerate genetic differentiation among demes (Roberts et al. 2013). Regardless of the nature of the barrier (one-way or two-way) populations isolated above barriers can experience a loss of genetic variability (Fagan 2002).

Fragmentation may also occur without hard barriers because of natural variation in habitat conditions or changes in relation to anthropomorphic habitat degradation (Fagan et al.

2002). While the effects of this form of fragmentation are less well understood in aquatic ecosystems (Newton et al. 2008), if viewed in a landscape resistance context (Ricketts 2001) long stretches of unsuitable habitat should lack stepping stone populations and may act as barriers that reduce connectivity and gene flow.

2.4 Population Size

The well-known relation between effective population size (N_e) and a population's response to evolutionary forces (i.e. genetic drift) makes an estimation of N_e an important goal for genetic analyses of threatened species. Population size, irrespective of its effect on genetic diversity, is closely tied to the probability of extinction and is therefore fundamental to the management of threatened and endangered species. Small size can make a population more vulnerable to extinction from random events associated with environmental or demographic stochasticity (Lande 1988, Lande et al. 2003, Jeppsson and Forslund 2012, Wootten and Pfister 2013). Outside of the obvious fact that fewer individuals need to perish in a small population to drive a species to extinction, random events have a proportionally larger impact on small populations. Positive density dependence can create another vulnerability for small populations, known as an Allee effect (Courchamp et al. 1999), which may cause smaller populations to decline at ever increasing rates. An example pertinent to freshwater mussels, which are broadcast spawners, would be a decline in fertilization rates due to a lack of suitable mates in close proximity.

Probability of extinction is also closely related to a species' geographic extent. A negative association between range size and extinction risk has been demonstrated in many

paleontological species assemblages (Jackson 1974; Hansen 1980; Stanley 1986; Jablonski 1986; Buzas & Culver 1991; Jablonski and Raup 1995, McKinney 1996) and some modern fauna (Şekercioğlu et al. 2004, Sodhi et al. 2008, Reside 2016). A widespread species may avoid extinction in a changing environment simply by having a higher likelihood of persisting somewhere in its range. As a result, widespread species are less likely to undergo a random walk to extinction.

Empirical evidence suggest local population size may interact with overall range size to influence extinction probability (Johnson 1998, Purvis et al, 2000). Species with small range size may be able to avoid extinction if they have large local population densities while species with low local population densities may persist if they have large range sizes. Therefore, extinction risk should be highest for species with both low local abundance and small range size. Local abundance should help to lower the risk of extinction from stochastic effects – large local populations would be more likely to weather random stochastic events. In contrast, large species range may produce a long-term advantage against extinction by providing widely dispersed populations that may be able to take advantage of shifting habitat availability over time.

2.5 Mussel Distribution and Habitat Associations

Central Texas is home to a diverse freshwater mussel fauna including three candidates for federal listing under the ESA - Texas Fatmucket (*Lampsilis bracteata*), Texas Pimpleback (*Quadrula petrina*), and Golden Orb (*Quadrula aurea*) - and one species, False Spike (*Fusconaia mitchelli*), that is awaiting status review. Historic distributions for these species included all or

parts of the Guadalupe and San Antonio River systems (Howells 2010; UFWS 2011) as well as other drainages in central Texas. However, recent evidence from surveys conducted post 1990 suggest all of these species have undergone moderate to severe range reductions.

Texas Fatmucket was never widely distributed in the Guadalupe and San Antonio systems, but is known to have ranged from northern Gonzales County upstream to Kerr County in the Guadalupe River system and from Bexar County upstream to the city of San Antonio in the San Antonio River system (Howells 2010; USFWS 2011). Recent surveys within the historic range of Texas Fatmucket have located a few small populations in the upper Guadalupe River and tributaries of the Colorado River basin (Howells 2010; Burlakova et al. 2010). Their continued existence in the lower Guadalupe and San Antonio systems is considered doubtful.

The Texas Pimpleback once ranged throughout most of the Colorado, Guadalupe, and San Antonio systems. Current data, however, suggest the Texas Pimpleback may now persist only in small populations in the San Saba, Concho, Guadalupe, and San Marcos Rivers (USFWS 2011). Surveys in the Guadalupe and San Antonio systems since 1992 have uncovered Texas Pimpleback populations in the Guadalupe River in Victoria (Howells 2005) and Gonzales counties (Randklev et al. 2011) and a single specimen in the San Marcos River (USFWS 2011). However, preliminary surveys for this research have documented the existence of several small populations of Texas Pimpleback in the San Marcos River near Luling, Texas and suggest this species may be more abundance than originally thought.

The False Spike once existed as two geographically distinct populations in the Rio Grande basin and in Central Texas, but the Rio Grande lineage appears to have died out prior to European settlement (Howells 2010). In Central Texas historical records indicate the False Spike

occurred in the Colorado, Guadalupe, and Brazos Rivers (USFWS 2011), but no live specimens had been documented since the 1970s (Howells 2010). The False Spike was presumed extinct (Howells et al. 1996; Haag 2009a) until a recent survey in the Guadalupe River in Gonzales County uncovered the first live population to be documented in over 30 years (Randklev et al. 2011). While this finding demonstrated the continued existence of the False Spike in central Texas, it is important to note that the specimens found were all adults in the same size class. Freshwater mussels can be long-lived with life spans ranging from <10 to approximately 130 years (Haag, 2009b) and functionally extinct populations, composed solely of non-reproducing adults, have been reported from heavily altered river systems (Layzer et al., 1993; Hughes and Parmalee, 1999). The lack of a range of size classes in the only known population of False Spike leaves the reproductive status of the species in question.

The historic range of the Golden Orb likely included all of the Guadalupe, San Marcos, San Antonio, and Nueces-Frio river drainages (Howells et al. 1996). However, recent research identified only a few populations in the Nueces-Frio drainage (pers. comm., Charles Randklev, Texas A&M University), including a lentic population in Lake Corpus Christi, and only a few live specimens from the upper Guadalupe (Howells 2006; Burlakova and Karatayev 2010). Taken together the evidence suggests the modern distribution of the species is largely confined to the lower sections of the Guadalupe, San Marcos, and San Antonio drainages. Despite this range reduction the Golden Orb is likely the most abundant of the four threatened freshwater mussel species in central Texas. For example, a longitudinal survey, conducted in 2011 by the San Antonio River Authority, documented the continued existence of the Golden Orb throughout

much of the lower San Antonio River (Larralde 2011). Other relatively healthy populations have been identified in the lower Guadalupe and San Marcos rivers (Burlakova and Karatayev 2010).

The lower sections of the Guadalupe and San Antonio river systems in south central Texas clearly harbor important populations of threatened freshwater mussel species. However, the south central region is among the fastest growing regions in Texas with an expected population increase of 75% and increased water demands of 32% by 2060 (TWDB 2012). Compounding the effects of growing water needs in the region is the potential for more severe weather events, due to changing climatic conditions, with the capacity to affect freshwater mussel habitat (Mishra and Singh 2010; Neilson-Gammon 2011). Environmental flows standards have been set for the Guadalupe and San Antonio systems (TCEQ 2012) as required by the Texas Legislature (Brown 2001; Armbrister 2005). Flows standards attempt to preserve functioning aquatic ecosystems by mimicking natural flow regimes, including seasonal and inter-annual variability. Standards prescribe minimum flow conditions for four flow categories – subsistence flows, base flows, pulse flows, and overbank flows (National Research Council 2005). Subsistence flows are defined as "…the minimum streamflow needed during critical drought periods to maintain tolerable water quality conditions and to provide minimal aquatic habitat space for the survival of aquatic organisms" (National Research Council 2005). While flow prescriptions are backed by extensive research, habitat conditions associated with most threatened freshwater mussel species are very general in nature (Howells et al. 1996; Howells 2010) and it is not clear how subsistence flow levels may affect mussel habitat. The lack of a clear understanding of the consequences of subsistence flows coupled with the likelihood of future severe drought events in Texas (Neilson-Gammon 2011) makes the collection of

quantitative data on the low-flow habitat characteristics associated with *healthy* mussel populations an important consideration for resource managers.

The requirements for listing a species under the ESA involve a comprehensive review of the species' biology, demographic status, habitat requirements, and the factors threatening its continued existence (USFWS 2011). Most freshwater mussels are of limited economic value and have received little scientific or regulatory attention. As a result, the information required for listing is limited for many rare mussel species in Texas (Howells et al. 1996; Howells 2010). Information that is available may be spatially limited due to the lack of public river access in Texas and the common practice of conducting surveys adjacent to publicly accessible locations such as public parks, road crossings, and municipal water facilities (Howells 2006; Howells 2005; Ford et al. 2010, Burlakova et al 2011). While state parks and road crossing provide ready access the reaches that are adjacent to these features may not provide optimal habitat for Unionid mussels or be representative of habitat conditions in the larger system. More to the point, the limited public access available in Texas represents a small percentage of the approximately 191,000 river miles encompassed by the state's 15 major river basins. Spatially restricted surveys may provide enough information to develop a relative measure of abundance (Burlakova et al. 2001), but may not be sufficient to 1) document the continued existence of highly threatened species and 2) estimate population levels on a river basin scale. The need to work in remote locations is underscored by the recent discoveries of extant populations of two species, Texas Fawnsfoot (*Truncilla macrodon*) (Randklev et al. 2010) and the previously discussed False Spike (Randklev et al. 2011), that were thought to be extinct in Texas (Howells et al. 1996; Haag 2009). Given these recent discoveries it is likely that other unknown

populations of rare freshwater mussels exist in the remote reaches of Texas' rivers. Research is needed to clarify the current distributional and demographic status of threatened freshwater mussels in Texas.

CHAPTER 3

METHODOLOGY

3.1 Mussel Surveys

Surveys for the presence of *Q. aurea* populations were conducted in the lower segments of the Guadalupe, San Antonio, and San Marcos rivers (Figure 3.1). Survey reaches typically began at public access points (i.e. road crossings, parks, or other public access point) and were accessed utilizing small watercraft (powered jon boat, canoe, or kayak). A preliminary search for sampling locations was performed during low-flow periods to facilitate locating mussels and was conducted at locations where habitat conditions or the presence of spent values signified a high potential for the occurrence of mussels. When mussels were encountered in the preliminary search the extent of the mussel bed was measured and delineated into separate habitat types (riffle, run, or pool). Surveys were then performed as qualitative timed searches and mussels were identified and collected through visual or tactile encounters while wading or using snorkeling equipment.

Sampling reaches were searched until a mussel bed of sufficient size (≥ 25 *Q. aurea* individuals 40 mm or larger) for collecting genetic material was encountered. The minimum sample size target of 25 was selected for two reasons: 1) very rare alleles (i.e. those found at frequencies <0.01) provide little information for most population-based analyses as their presence may be due to recurrent mutations rather than historical association or contemporary gene flow (Hartl and Clark 1997) and 2) simulation studies of microsatellite-based analyses have demonstrated that a sample of 25 to 30 individuals is sufficient to obtain all informative alleles at frequencies that are representative of the population allele frequencies (Hale et al. 2012).

We achieved our target sample size in all locations except for the Runge population in the San Antonio River where only 22 specimens of suitable size were found.

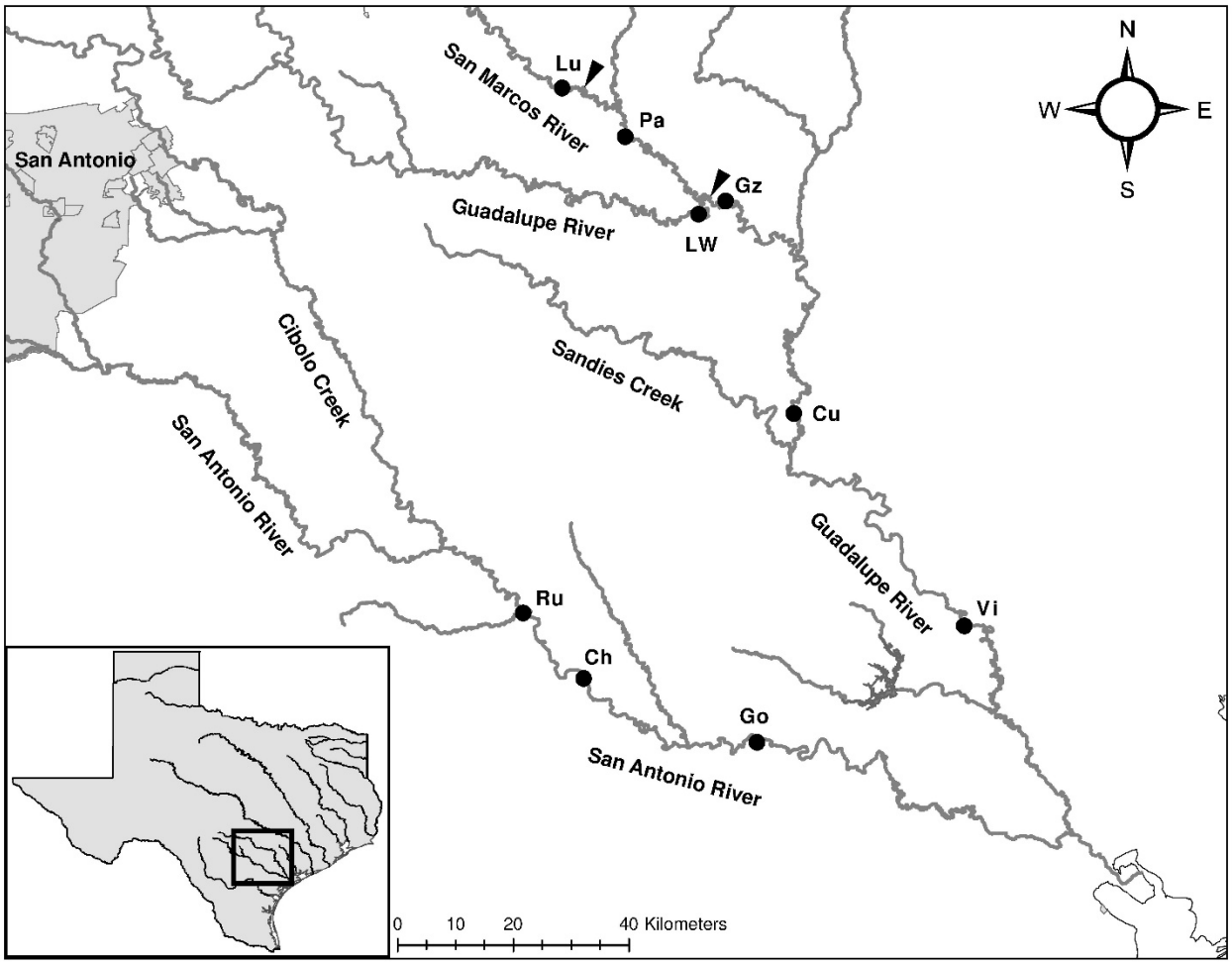

Figure 3.1. *Quadrula aurea* genetic sampling sites (black circles) in the San Antonio, Guadalupe, and San Marcos Rivers. Sampling site names are abbreviated as: Ch, Charco; Cu, Cuero; Go, Goliad; Gz, Gonzales; LW, Lake Wood; Lu, Luling; Pa, Palmetto; Ru, Runge; Vi, Victoria. Black wedges indicate the location of large dams.

All *Q. aurea* individuals encountered during surveys were extracted from the sediment and retained for enumeration and genetic sampling. Specimens were held in a mesh bag which was submerged in moderate flow until processing. Since shell length is an indicator of relative age in freshwater bivalves (Harmon and Joy, 1990, Haag, 2009b), all *Q. aurea* individuals collected were measured with calipers to the nearest 0.1 millimeter along their long axis to

develop information on relative age structure of mussel beds. All other threatened freshwater mussel species encountered during surveys were counted in place and left undisturbed or extracted from the sediment to verify species identifications and take length measurements. Only a select number of other threatened freshwater mussel species were measured to determine overall size range. After processing mussels were returned to the bed and placed in the substrate with their posterior siphons exposed.

3.2 Habitat Measurements

A set of physical variables commonly assessed in riverine studies (i.e., wetted channel width, channel slope, flow velocity, water depth, and substrate composition) were measured at each mussel bed where genetic samples were collected (Table 3.1). Water velocity and depth measurements were then used to estimate a set of complex hydraulic variables (Table 3.2) that have been shown to interact with substrate composition to influence the suitability of mussel habitat (Hardison and Layzer, 2001; Gangloff and Feminella, 2007; Zigler et al., 2008; Allen and Vaughn, 2010). The particle size distribution from the sediment sample was used to develop an estimate of relative shear stress (RSS) to evaluate substrate stability under the flow conditions present at the time of sampling. RSS is defined as the ratio of the friction force acting on the substrate (shear stress) to the friction force required to set a given particle size in motion (critical shear stress; Morales et al., 2006). Habitat patches that experience RSS values ≥1 during spates are considered unstable and represent poor quality mussel habitat (Morales et al., 2007; Allen and Vaughn, 2010).

Table 3.1. Description of habitat variables measure at mussel sampling locations or calculated through the GIS analysis.

Parameter	Abbreviation	Description
Bed Width	BW	Width of mussel bed
Wetted Width	WW	Width of wetted region of channel
Bankfull Width	BFW	Width of wetted region of channel at bankfull flow
Average Depth	AD	Mean depth across transect
Average Bankfull Depth	ABFD	Mean depth across transect at bankfull flow
Gradient	Grad	Slope of channel at mussel bed
Average velocity at 60% depth	Vel60	Mean flow velocity across transect at 60% of depth
Average velocity near bed	VelNB	Mean flow velocity across transect 5 cm from bottom
D16	D16	Sediment particle size where 16 % of sample is smaller
D50	D50	Sediment particle size where 50 % of sample is smaller
D84	D84	Sediment particle size where 84 % of sample is smaller
Geometric mean	GeoM	Geometric mean of sediment particle sizes for sample
Relative shear stress	RSS	Complex hydrologic variable (see Table 3.2)
Boundary Reynolds number	Re	Complex hydrologic variable (see Table 3.2)
Froud number	Fr	Complex hydrologic variable (see Table 3.2)
Percent landuse as developed	Devlp	NLCD data classes 21, 22, 23, and 24
Percent landuse as forest	Forest	NLCD data classes 41, 42, and 43
Percent landuse as agricultural land	Agricul	NLCD data classes 81 and 82
Percent landuse as shrubland	Shrub	NLCD data class 52
Percent landuse as herbaceous	Herb	NLCD data class 71
Soil composition s7265	s7265	STATSGO2 soil classification s7265
Soil composition s7462	s7462	STATSGO2 soil classification s7462
Soil composition s7718	s7718	STATSGO2 soil classification s7718
Soil composition s7719	s7719	STATSGO2 soil classification s7719
Soil composition s9710	s9710	STATSGO2 soil classification s9710

A Marsh-McBirney™ Flo-Mate flowmeter (Marsh-McBirney, Frederick, Maryland) and a 1.5 m top setting wading rod were used to measure water velocity and depth at five equally spaced locations (0.1, 0.3, 0.5, 0.7, and 0.9× channel width) along a single transect bisecting the center of the mussel bed. At each transect point we measured water velocity at 60% of depth

and again at 5 cm above the substrate to characterize near-bed velocity. A survey level and stadia rod were used to measure the change in water surface elevation (channel slope) over the length of the mussel bed. Substrate composition tended to be relatively uniform across the majority of mussel beds in this study, therefore, we collected a single sediment core (16.5 cm diameter x 8 cm deep) from the center of the mussel bed with a custom designed sampler constructed of PVC pipe and designed to work in course (gravel to cobble) substrate. The sediment sample was returned to the University of North Texas where it was oven dried at 110° F for 24 hours, ground with a mortar and pestle to separate aggregated particles, and sieved to partition substrate size fractions. Sieve size fractions were 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.063 mm. After separation each size fragment was weighted to the nearest 10th of a gram. Sediment particle size classes and complex hydraulic variables, described in Tables 3.1 and 3.2, were calculated using formulas from Allen and Vaughn (2010) and references therein. Values for each hydraulic variable at each of the 5 transect points were calculated using the near-bed velocity measurements. We took the arithmetic average of shear stress (τ) as an estimate of mean shear stress ($\bar{\tau}$) for the mussel bed. Mean shear stress was compared to the critical shear stress (τ_c) estimate for the median particle size (D_{50}) to develop an estimate of RSS for the entire mussel bed. In the calculation of critical shear stress a value of 0.06 was used for the dimensionless Shield's parameter (θ_c) at sites where the substrate was composed of packed gravel and sand with an armoring of larger sized particles on the surface (Gordon et al., 2004). At sites composed primarily of fine substrate (sand and/or silt) a value of 0.04 was used for the Shield's parameter.

Table 3.2. Description of measured substrate and estimated hydraulic variables used to characterize habitat conditions. D_x = substrate particle size (cm) at which x% of the sample by mass is finer, U = mean water velocity (cm/s), d = water depth (cm), n = sample size (5), g = acceleration due to gravity (980 cm/s), v = kinematic viscosity of water (0.01 cm2/s), p = density of water (0.998 g/cm3), p_s = density of substrate (2.65 g/cm3), θ_c = Sheild's parameter (0.07) (Gordon et al., 2004).

Variable Description: Substrate Variables	Formula	Description	Source
Median particle size (D_{50}), cm	D_{50}	Median particle size of sample	Gordon et al., 2004
Mean particle size ($\overline{D}$), cm	$\frac{D_{16}+D_{50}+D_{84}}{3}$	Mean particle size of sample	Gordon et al. 2004
Bed roughness (k_s), cm	$3.5 \times D_{84}$	Topographical variation of streambed	Rempel et al., 2000
Hydraulic Variables			
Froude number (Fr), dimensionless	$\frac{U}{(gd)^{0.5}}$	Ratio of inertial to gravitational forces in flow	Statzner et al., 1988
Boundary Reynolds number (Re_*), dimensionless	$\frac{U_* k_s}{v}$	Ratio of internal to external turbulent forces	Statzner et al., 1988
Shear velocity (U_*), cm/s	$\frac{U}{5.75 log_{10}\left(\frac{12d}{k_s}\right)}$	Friction velocity	Statzner et al., 1988
Shear stress (τ), dynes/cm^2	pU_*^2	Force of friction on substrate	Statzner et al., 1988
Mean shear stress ($\bar{\tau}$), dynes/cm^2	pU_*^2/n	Mean of point shear stress estimates	Adapted from Statzner et al., 1988
Critical shear stress (τ_c), dynes/cm^2	$\theta_c g D_{50}(p_s - p)$	Force required to initiate motion of median particle size	Gordon et al., 2004
Relative shear stress (RSS) dimensionless	$\frac{\bar{\tau}}{\tau_c}$	Ratio of mean shear stress to critical shear stress	Adapted from Morales et al., 2006

Land cover and soil conditions within sub-catchments above each *Q. aurea* sampling site were evaluated using a GIS approach. Determination of sub-catchment characteristics was achieved using ArcGIS software (v 10.4.1, Environmental Systems Research Institute, Inc., Redlands) and a common procedure to define the catchment area contributing flow to a specific location. Genetic sampling sites were used as pour-points and catchment boundaries were determined using digital elevation models (DEMs) acquired from the USGS National Elevation Dataset website (https://nationalmap.gov/elevation.html). DEMs were processed

through a series of ArcMap tools (Fill, Flow Direction, Flow Accumulation, and Watershed) to define the area of land which drains to each sampling site (i.e., catchments). Sub-catchments were then created by clipping catchments at a 10 mile radius. Restricting sub-catchments to a distance of 10 miles was done to 1) evaluate the potential for local land cover factors to effect genetic diversity/structure and 2) facilitate variation among sites that exist in the same drainage basin and would otherwise exhibit the same overall catchment conditions if the full catchment was used. Land cover in each sub-catchment was quantified by clipping the USGS 2011 National Land Cover Dataset (https://www.mrlc.gov) to the 10 mile sub-catchments and then calculating the percentage of each land cover category contained within the sub-catchment (Figure 3.2). Soil data was obtained from the United States General Soil Map (STATSGO2; https://data.nal.usda.gov/dataset/united-states-general-soil-map-statsgo2) and process in the same manner as the land cover (Figure 3.3).

The relations among variables were evaluated using the raw data in a Spearman correlation analysis using the program Corrplot (Wei and Simko 2017) in the R statistical environment (R Core Team 2016). Variation in habitat variables among river basins was analyzed under a three basin model (i.e., Guadalupe, San Marcos, and San Antonio) and a two basin model that combined data from the Guadalupe and San Marcos basins under the assumption that *Q. aurea* in those basins represent a single panmictic population. Habitat differences among basins under the three basin model were assessed using a Kruskel-Wallis rank sum test (Hollander and Wolfe 1973). Significant results under the Kruskel-Wallis test were further analyzed for differences between specific basins using Dunn's post-hoc multiple comparison of rank sums test (Dunn 1964) with correction for multiple comparisons using the

procedure of Benjamini and Hotchberg (1995). The comparison of habitat differences under the two basin model was achieved using a Wilcoxon signed-rank test. All statistical testing of habitat variable was carried out in the R statistical environment (R Core Team 2016).

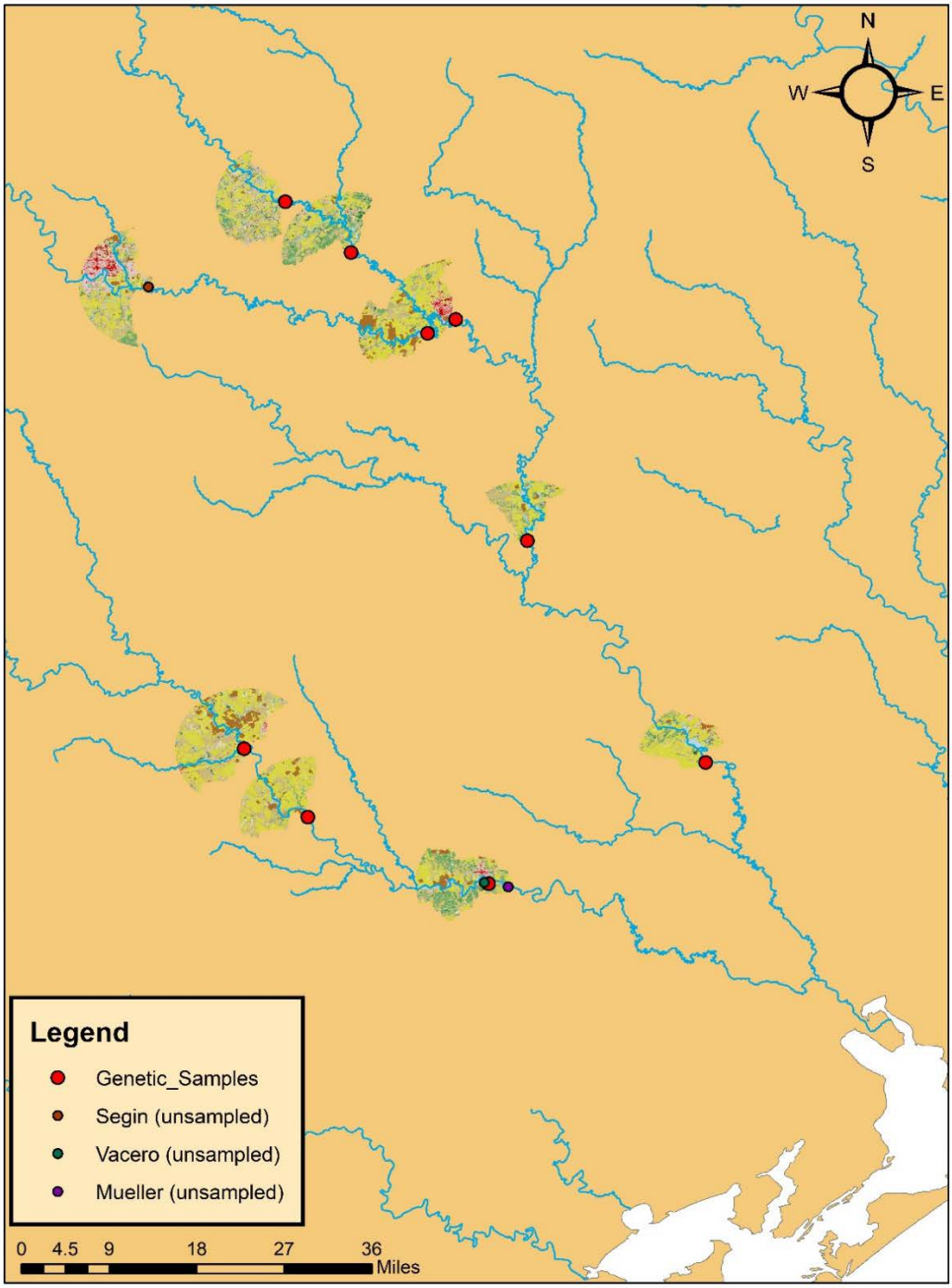

Table 3.3. Map of drainage specific landuse within 10 kilometers of *Quadrula aurea* sampling locations.

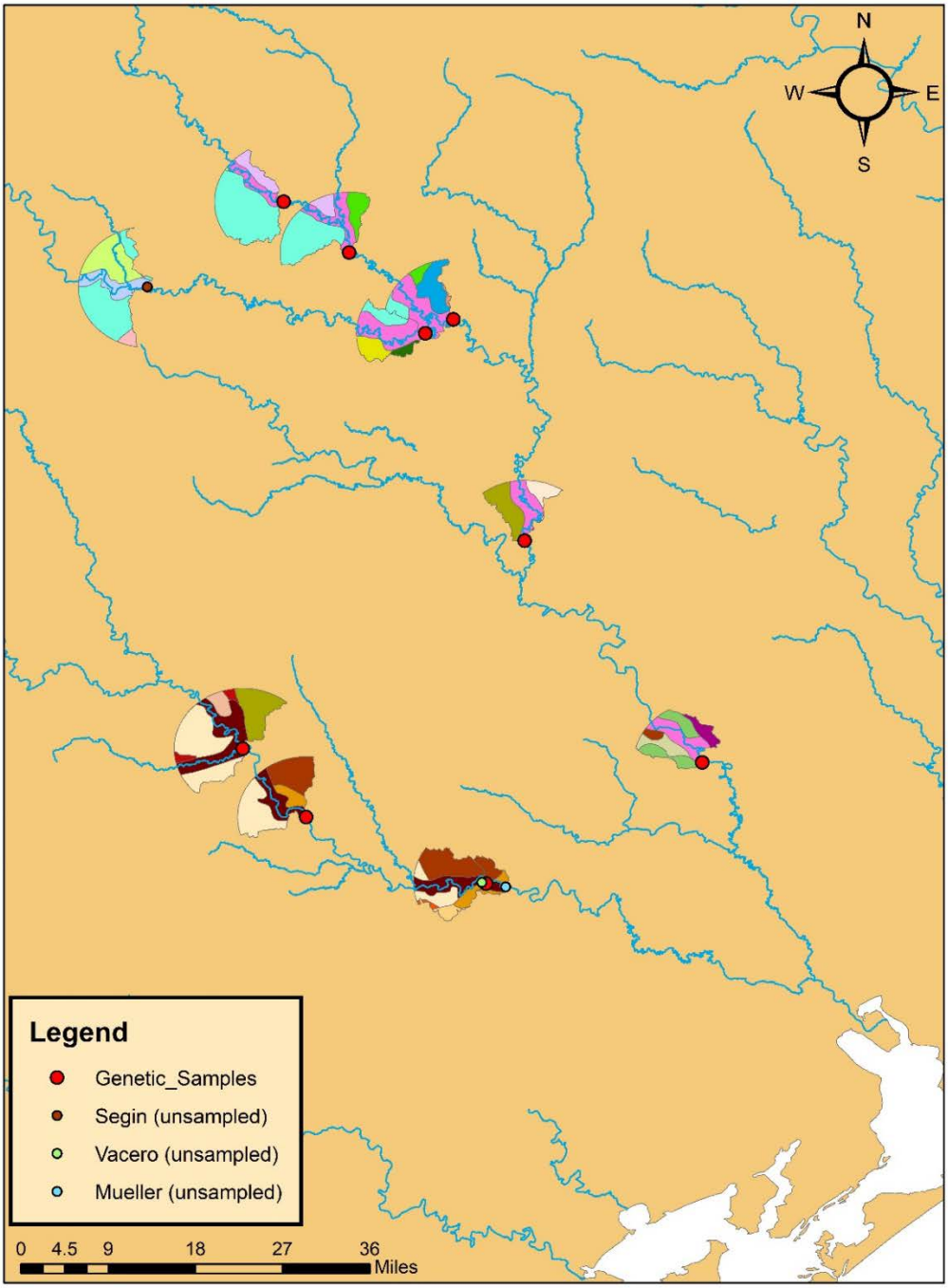

Table 3.4. Map of drainage specific soil composition within 10 kilometers of *Quadrula aurea* sampling locations.

3.3 Genetic Sample Collection and Processing

Samples for genetic analysis were collected from 258 *Q. aurea* individuals at nine

spatially discrete locations (Figure 3.1). Tissue collection was accomplished with a minimally invasive integument swabbing technique (Henley et al. 2006) where surface tissue cells were gathered from the lower visceral mass and foot with a small cytology brush. Mussels were placed in a wire basket in flowing water and allowed to resume normal siphoning behavior. A small wooded wedge was place between the gaping valves and used to gently force the vales apart and allow the insertion of a small cytological brush (~10 mm). Utilization of this technique avoids the potential for damage that exists when attempting to pry apart tightly closed valves. Swab sampling was conducted with commercially available kits (Qiagen Inc., Valencia CA) designed for the collection of buccal (cheek) cells and was restricted to mussels of at least 40 mm in length to avoid damage to small specimens.

Genomic DNA was extracted from integument swabs following manufacturer protocols for buccal cell samples with the Gentra® Buccal Cell extraction kit (Qiagen 2011). Briefly, cells were lysed at 55°C for 24 hours in an anionic detergent with 1 µL Proteinase K added to deactivate intracellular DNases and stabilize the DNA. A salt precipitation procedure was used to remove proteins from solution and purified DNA was recovered through ethanol precipitation and rehydrated in a solution of 1mM EDTA and 10mM Tris-Cl at pH 7.5. Recovered DNA concentration was assessed using UV absorption spectroscopy (Fasman 1975) and a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

3.4 Primer Testing

Genetic material collected from 34 individuals in a single mussel bed in the San Antonio River near Goliad, Goliad County, TX was used to test for cross-species amplification in *Q.*

aurea. A set of 27 microsatellite primer pairs (see Table 4.2), previously tested in the congeneric mussel species *Q. fragosa* (Hemmingsen et al. 2009; pers. comm., Kevin Roe, Iowa State University) and *Amphinaias pustulosa* (pers. comm., Kevin Roe, Iowa State University), were tested for their ability to amplify *Q. aurea* DNA via polymerase chain reaction (PCR). PCRs were done in a 10 µL solution containing approximately 45 ng of genomic DNA, 0.4 µM of both 5' and 3' primers, 0.4 mM of dNTPs, 2 mM of $MgCl_2$, and 1× PCR buffer, and 0.5U of Taq DNA polymerase. Thermal conditions included 94 °C for 2 min; 32 cycles of 94 °C for 40 sec, primer specific annealing temperature for 40 sec (see Table 4.5), 72 °C for 40 sec; a final extension step at 72 °C for 3 min, and a hold at 4 °C until removal from the thermocycler. Visualization of PCR products and determination of successful amplification were achieved by gel electrophoresis in a 1% agarose gel with ethidium bromide. Loci that amplified successfully were assessed for polymorphism by sequencing a minimum of six individuals using Big Dye® terminator sequencing (Thermo Fisher Scientific, Waltham, MA) and an Applied Biosystems® 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). All loci that amplified consistently and displayed polymorphism were genotyped in the complete sample set of 34 individuals using fluorescently labeled primers. Genotyping results were visualized and allele sizes determined using GeneMarker software (SoftGenetics, State College, PA).

Loci were checked for the presence of genotyping errors (null alleles, stuttering, or large allele dropout) using Micro-Checker v 2.2.3 software (Van Oosterhout et al. 2004), which is described more fully in section 3.4. We tested for deviation from Hardy-Weinberg equilibrium and linkage disequilibrium using the software program Arlequin v 3.5.1.2 (Excoffier and Lischer

2010). Significance values were adjusted for multiple comparisons by controlling for the false discovery rate using the approach of Benjamini and Hotchberg (1995).

3.5 Power Analysis of Microsatellite Markers

The final set of unambiguous polymorphic microsatellite markers were tested, in association with the overall sample design, for their power to identify significant genetic differentiation among populations of *Q. aurea* using the computer program POWSIM (Ryman and Palm 2006). POWSIM applies a simulation approach to estimate the statistical power of a given set of genetic and sample parameters to test the null hypothesis (H_0) of identical allele frequencies among populations at a given value of true genetic divergence. Briefly, the model assumes a base population of infinite size is segregating for a defined number of selectively neutral loci with user defined allele frequencies. The base population is divided into *s* subpopulations of equal effective size (N_E) through random sampling of $2N_E$ genes (corresponding to N_E diploids). Each subpopulation is then allowed to drift for *t* generations and the expected amount of divergence among populations in generation *t* is quantified by:

$$F_{ST} = 1 - (1 - 1/2\,N_E)^t$$ (Nei 1987).

In generation *t* a random sample of 2*n* genes is drawn (with replacement) from each locus from each subpopulation, and the H_0 of identical allele frequencies in all the *s* populations is tested with Fisher's exact test using a Markov Chain Monte Carlo procedure (Raymond and Rousset 1995). The entire simulation process is repeated a large number of times (runs) and the proportion of significant outcomes ($P<0.05$) represents the estimate of statistical power.

Estimates of the type I error (α error) rate can also be obtained by using samples drawn directly from the base population and omitting the drift steps (i.e. true F_{ST} = 0).

Table 3.5. Running parameters for the POWSIM simulation used to perform the power analysis.

	Number of loci	6
	Number of populations	9
	Effective size (N_E) when drifting apart	500
	Generations of drift	10
	Sample size per population	25
	Number of simulation runs	1000
Parameters for Fisher's Exact Test	Burn in	1000
	Number of batches	100
	Iterations per batch	1000

Allele frequencies used in the POWSIM analyses were calculated from the 34 Guadalupe samples genotyped during primer testing (see section 3.3). Other parameters (Table 3.3) were defined to reflect the basic design of the full genetic analysis (i.e., the six loci identified in the primer testing, the nine sampled populations, and a minimum sample size of 25). Simulation parameters (i.e., N_E when drifting apart and number of generations of drift; Table 3.3) were designed to achieve a true F_{ST} of 0.01. In other words, the power analysis defined above represents an estimate of the statistical power of the overall study, using six microsatellites with given levels of polymorphism, to detect a true genetic divergence among populations of F_{ST} = 0.01.

3.6 Analyses of Population Genetic Structure

Six microsatellite loci (Polymorphic loci in Table 4.5) identified in the primer testing

phase and previously described in the congeneric species *Quadrula fragosa* (Hemmingsen et al. 2009; Roe 2010) were used to define genetic multilocus genotypes for *Q. aurea*. Polymerase Chain Reaction (PCR) amplification of microsatellite loci was performed in a 10 µL reaction volume with approximately 45 ng of genomic DNA, 0.4 µM of forward and reverse primers, 0.4 mM of each dNTP, 2.0 mM of $MgCl_2$, 1X PCR buffer, and 0.5U of Taq DNA polymerase. Forward primers were tagged on the 5' end with one of three possible fluorescent dyes (6-FAM, NED, or HEX). Reactions were carried out under the following thermal conditions: 94 °C for 2 min; 32 × [94 °C for 40 sec; primer specific annealing temperature for 40 sec; 72 °C for 40 sec]; 72 °C for 3 min. Visualization of PCR products and determination of successful amplification were achieved by gel electrophoresis in a 1% agarose gel with ethidium bromide. PCR products were then run on an Applied Biosystems® 3130xl Genetic Analyzer, and allele sizes were determined using GeneMarker software (SoftGenetics, State College, PA).

3.6.1 Detection of Genotyping Errors

Microsatellite genotypes were checked for the presence of genotyping errors with the software program MICRO-CHECKER (Van Oosterhout et al. 2004), which estimates probabilities for the observed numbers of homozygotes in each allele size class by comparing observed genotypes to a distribution of expected genotypes developed through a randomized Monte Carlo process. Significant deviations from expected distributions are interpreted as evidence for genotyping errors due to null alleles, short allele dominance, or stuttering. Any locus exhibiting an observed homozygosity value outside of the Bonferroni corrected 95% confidence interval generated by the simulation was determined to be affected by genotyping errors.

3.6.2 Genetic Diversity

Microsatellite genotypes for each population were tested for deviations from Hardy-Weinberg equilibrium (HWE) using the program Arlequin v. 3.5.1.2 (Excoffier and Lischer 2010) and a Markov-chain based exact test (Guo and Thomson 1992) with a chain length of 2×10^6 and 10^5 dememorization steps. Pairwise independence between loci (linkage disequilibrium) was assessed using Fisher's exact test with the program GENEPOP v. 4.3 (Rousset 2008). Probability estimates for HWE and linkage disequilibrium were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) method for control of the false discovery rate. Genetic diversity within populations was characterized in terms of numbers of alleles per locus (A), allelic richness (A_R) after rarefaction to the smallest sample size (Hurlbert 1971), observed (H_O) and expected (H_E) heterozygosity, and the inbreeding coefficient (F_{IS}). The program FSTAT v. 2.9.3.2 (Goudet 1995) was used to calculate both A and A_R. Nei's unbiased estimate of H_E (Nei 1987) was calculated using Arlequin v. 3.5.1.2 (Excoffier and Lischer 2010) and F_{IS} was calculated with the program GDA v. 1.0 (Lewis and Zaykin 2001). Both H_E and F_{IS} were calculated for each locus and population and then averaged across loci to calculate a mean value for each population. Bootstrapping with 10^4 replications was applied in GDA to estimate 95% confidence intervals for F_{IS} estimates. Statistical differences in genetic diversity measures among populations were assessed with Kruskal-Wallace nonparametric analysis of variance tests.

3.6.3 Genetic Structure

Genetic differentiation among populations was quantified using three separate estimators, F_{ST}, R_{ST}, and the unbiased version of Jost's D (D_{est}). All calculated p values were

adjusted for multiple comparisons using the Benjamini and Hochberg (1995) method.

Pairwise F_{ST} estimates were calculated in the statistical program Arlequin v. 3.5.1.2 (Excoffier and Lischer 2010) using an analysis of molecular variance framework (AMOVA; Excoffier et al. 1992) and the statistical approach of Weir and Cockerham (1984). An AMOVA was also used to investigate the partitioning of genetic variation at three hierarchical levels: across all populations, among populations within each drainage basin, and between drainage basins. Hierarchical AMOVAs were conducted using data pooled across loci and with each locus individually to determine if results were consistent among loci. Preliminary analysis suggested little genetic differentiation between populations in the Guadalupe and San Marcos basins. Therefore, the Guadalupe and San Marcos basins were combined for the hierarchical AMOVA procedure under the assumption that they comprise a single panmictic population. Statistical significance of *F*-statistics was tested by permuting genotypes among populations and populations among groups of populations 10^5 times to develop null distributions for comparison to the observed data.

The F_{ST} analogue R_{ST} (Slatkin 1995) may be more appropriate for microsatellites that follow a step-wise mutation model. Global and pairwise estimates for R_{ST} were calculated using Arlequin v. 3.5.1.2 (Excoffier and Lischer 2010). The approach of Hardy et al. (2003) was then employed to evaluate the relative importance of stepwise mutation in the estimation of population differentiation among *Q. aurea* populations. The Hardy et al. (2003) method compares the observed value of R_{ST} to its mean expected value (pR_{ST}), which is calculated by permuting the different allele sizes found at each individual locus among allelic states. An observed R_{ST} value significantly larger than the pR_{ST} suggests mutations contribute to genetic

differentiation (i.e., mutation ≥ migration) and that loci follow, at least partially, a stepwise mutation model. The program SPAGeDi v 1.5 (Hardy and Vekemans 2002) was used to compare observed values of R_{ST} to mean estimates of pR_{ST} calculated with 10^4 permutations.

The value of F_{ST} as a measure of genetic distance among populations can be limited with microsatellite data when within-population variation is large. Because among-population variation and total genetic variation are not independent the value of F_{ST} is constrained by the level of within-population variation. If genetic variation is large within populations F_{ST} values can be quite low even when there is significant among-population variation. To address this issue Jost (2008) derived an alternative measure of population differentiation (*D*) based on ecological diversity theory and the effective number of alleles. *D* is designed to scale linearly with differences among populations; if two subpopulations consist of *k* equally common alleles *D* will represent the proportion of alleles that are unique to each subpopulation. Jost's unbiased measure of genetic distance D_{est} (Jost 2008) was calculated using the DEMEtics (Gerlach et al. 2010) package in the R statistical environment (R Core Team 2014).

3.6.4 Assignment Test

The program STRUCTURE (Pritchard et al. 2000) was used to test the distinctiveness of spatially discrete populations. STRUCTURE is a model based clustering algorithm that identifies subgroups with distinct allele frequencies. The program assumes a set of *K* populations and individuals are assigned to a population or jointly to two or more populations if their genotypes indicate admixture. STRUCTURE was run for $K = 1, 2, \ldots 9$ populations with a burn-in period of 10^4 repetitions, 5×10^4 repetitions for the MCMC run, and a model that assumed admixture. The

default setting for STRUCTURE uses only the genetic data to estimate population structure while the LOCPRIOR setting, which uses sampling locations as prior information, can assist the clustering process when genetic structure is relatively weak (Hubisz et al. 2009). STRUCTURE was run under both the default setting and the LOCPRIOR setting to assess the relative ability of the program to detect genetic structure.

Detection of the true *K* in STRUCTURE is based on the posterior probability of the data (LnP(D)) for a give *K*, calculated by taking the mean of the log likelihood functions at each step in the Markov chain Monte Carlo (MCMC) procedure and subtracting half their variance. In theory, the maximal value of LnP(D) indicates the true value of *K*. However, simulations have shown that LnP(D) values may not show a clear mode and may continue to increase slightly after the true *K* is reached (Evanno et al. 2005). To supplement our model selection process we applied the ad hoc statistic Δ*K* (Evanno et al. 2005), which tracks the second order rate of change in the LnP(D) and will show a clear inflection were LnP(D) is maximal and the variance between runs is minimal. Individual membership coefficients (Q) were averaged across individuals within each population to calculated population specific membership coefficients ($\hat{Q}$) for each K.

3.6.5 Spatial and Environmental Influence on Genetic Structure

Two approaches were utilized to investigate the relative influence of geographic distance and environmental distance on genetic structure. The first approach used a mantel test to compare matrices of pairwise D_{est} and pairwise river distance to evaluate weather *Q. aurea*'s genetic population structure conformed to an IBD model. Mantel tests were conducted

in the software program PASSaGE v. 2.0.11.6 (Rosenberg and Anderson 2011) and tested for significance by randomly permuting the order of elements in one matrix 10^5 times to develop a null distribution for comparison to the observed data.

The second approach used the R statistical environment (R Core Team 2014) and the program Sunder (http://www2.imm.dtu.dk/~gigu/Sunder) to quantify the relationship between spatial and environmental factors and genetic covariance. Sunder summarizes genetic variation among populations with a covariance matrix model which is then tested against independent matrices based on geographic distance, environmental distance, and the combination of geographic and environmental distance. The underlying assumption is that genetic covariance decays in an exponential fashion as a function of geographic and environmental distances.

Model parameters include a vector of unknown parameters $\theta = (\alpha, \beta_D, \beta_E, \gamma, \delta)$ that define various aspects of the model. The two parameters β_D and β_E quantify the magnitude of the effect of geographic and environmental distances, respectively, on genetic covariance. The other parameters are: α which controls the variance of allele frequencies under a Dirichlet distribution, γ which is an adimensional factor that quantifies the smoothness of spatial variation of the allele frequencies, and δ which controls the magnitude of the "nugget effect" (a geostatistical approach to allow for large discontinuity in the covariance structure and account for large genetic differences between geographically close populations). The posterior probabilities for θ and its components are inferred using a Markov Chain Monte Carlo (MCMC) simulation with upper bounds for component priors that are large enough so that their values have no effect on posterior distributions.

Sunder also provides a model selection procedure that facilitates selection of the model (geographic, environmental, or both) that best fits the genetic covariance model. Model selection is achieved with a cross-validation procedure that splits the dataset into a training set (a random subset of locations × loci) and a validation set (the remaining data points). The training sub-set is used to estimate the parameters under the three sub-models. Parameter estimates are then included in a likelihood function designed to evaluate the probability of the validation set. Estimation and evaluation of the likelihood for the validation set are performed for all three sub-models and the model achieving the highest probability is selected.

A geographic distance matrix for use in the Sunder analysis was created by measuring the river-distance between all sampling sites in ArcMap (v 10.4.1, Environmental Systems Research Institute, Inc., Redlands). Several environmental distance matrices were created from the habitat data measured during mussel survey efforts or calculated using GIS analysis (described in section 3.2). The degree of correlation between habitat variables was assessed with a Spearman correlation matrix. The complete suite of environmental variables was then loaded into the software program PRIMER-E v 6 (Clarke and Gorley 2006), normalized using the equation $x - \bar{X}/SD$, and then evaluated with a Principle Components Analysis. Variables with the highest loading coefficients in the first two principle components were selected for further analysis. If two variables with high loading coefficients were highly correlated the variable that represented a unique habitat category not already present in the dataset was selected. Habitat variables selected through the PCA analysis were then used to calculate a Euclidian distance based environmental distance matrix. Two other distance matrices were created to specifically reflect land cover characteristics and soil types within 10 kilometers of each sampling location.

3.7 Demographic History and Effective Population Size (N_e)

Two methods were used to investigate the demographic history of each population. A rapid decline in effective population size (N_e) will reduce the number of alleles quicker than observed heterozygosity values (Maruyama and Fuerst 1985). Consequently, populations that have experienced a recent size reduction will exhibit an excess of heterozygosity relative to HWE. We tested for the effects of recent population decline using the simulated coalescence approach of Cornuet and Luikart (1996) implemented in the program BOTTLENECK (Piry et al. 1999). Differences between H_O and H_E were assessed under three mutation scenarios: the Infinite Alleles Model (IAM), a Stepwise Mutation Model (SMM), and a Two-Phase Model (TPM). Significance was determined with a Wilcoxon sign-rank test, which should provide the highest statistical power given the number of loci used in this study (Piry et al. 1999).

The second approach used the Bayesian coalescent-based method implemented in MSVAR 1.3 (Storz and Beaumont 2002) to perform a more detailed investigation of the demographic history of each population. MSVAR is a model based Bayesian full-likelihood method that uses allele frequency data to detect, quantify, and date changes in N_e. The model assumes a closed population that changed from the ancestral population size (N_1) to the current population size (N_0) over time X_a. Calculating X_a in terms of years requires an estimate of the generation time (γ) for the species in question ($X_a = \gamma \times t_a$ where t_a is number of generations since the population began to change). No information is available for the generation time of *Q. aurea*, but information is available for *Q. pustulosa* (Haag and Staton 2003), which is considered to be closely related to *Q. aurea* (Serb et al. 2003). Haag and Staton (2003) found some variation in the age of reproductive maturity in *Q. pustulosa*, but report that

more than 50% of the individuals sampled at 5 years of age were reproductively mature. Therefore, model estimates of X_a for *Q. aurea* were based on a generation time (γ) of 5 years.

Within MSVAR, change in population size can be modeled as linear or exponential and mutations are assumed to follow a strict single step mutation model at a rate μ. The model produces posterior probability distribution estimates forN_0, N_1, X_a, and μ using MCMC simulation. Models must be supplied with estimated prior distributions for all modeled parameters, which are assumed to be log-normal. The means and standard deviations of these log-normal distributions are themselves drawn from prior (or hyperprior) distributions which are assumed to be normally distributed.

Wide uninformative priors and variation in the beginning population size (N_1; Table 3.2) were used to reduce the effect of priors on posterior distributions. Four or five independent chains were run for each set of priors assuming an exponential model with 10^{10} steps per run and a thinning interval of 10^5 steps. To eliminate potential bias on parameter estimation from starting values the first 10^4 samples were discarded to create a final sample size of 9.0×10^4.

Table 3.6. Parameters (priors and hyperpriors) for the MSVAR simulations. Starting values are the prior means and variances (in parentheses) for parameters. Hyperpriors are the means and variances (in parenthesis) for the prior means and variances.

	Priors; mean (variance)				Hyperpriors; mean (variance)			
	log(N_0)	log(N_1)	log(μ)	log(X_a)	log(N_0)	log(N_1)	log(μ)	log(X_a)
Priors 1	5 (2)	5 (2)	-4 (1)	5 (2)	4 (2) 0 (0.5)	5 (2) 0 (0.5)	-3.5 (0.25) 0 (0.5)	5 (3) 0 (0.5)
Priors 2	5 (2)	5 (2)	-4 (1)	5 (2)	4 (2) 0 (0.5)	3 (2) 0 (0.5)	-3.5 (0.25) 0 (0.5)	5 (3) 0 (0.5)
Priors 3	5 (2)	5 (2)	-4 (1)	5 (2)	4 (2) 0 (0.5)	4 (2) 0 (0.5)	-3.5 (0.25) 0 (0.5)	5 (3) 0 (0.5)

The MCMC approach attempts to create a stochastic Markov process that has a stationary distribution that matches the posterior distribution of interest. When the generated

Markov chain reflects the posterior distribution of interest it is said to have "converged". Unfortunately, convergence is not clearly defined for any MCMC run and the point at which it is reasonable to assume the samples are representative of the underlying stationary distribution of the Markov chain is not clear. Moreover, the Markov nature of the algorithm means that autocorrelation among samples will slow mixing, limit sampling from the entire stationary distribution, and potentially bias posterior distribution estimates. To assess chain convergence the Gelman-Rubin statistic (Gelman and Rubin 1992) was calculated for each independent run. The Gelman-Rubin approach for assessing convergence uses multiple chains to calculate an estimate of the posterior distribution and a scale reduction factor that reflects how much sharper the distributional estimate might become if the simulation were to run indefinitely. Chains were considered to have converged well when the Gelman-Rubin statistic was 1.1 or less (Gelman and Hill 2007). The overall performance of the MCMC process was assessed by calculation the degree of autocorrelation among samples at the 75^{th} quantile. All MCMC assessment statistics were calculated using the R statistical environment (R Core Team 2014).

Effect sizes, calculated as the unbiased standardized mean difference (*g*; Hedges and Olkin 1985; Cohen 1988), and the estimation of their 95% confidence intervals were used to assess the magnitude, precision, and statistical significance of any demographic change identified in the MSVAR process. The statistic *g*, in this context, is defined as the mean standardized difference between the log of the ancestral population size ($\log N_1$) and the log of the current population size ($\log N_0$). Standardization is achieved by dividing the mean difference by the pooled standard deviation (formulas in Appendix A). Effect sizes of each independent run were pooled to calculate a mean effect size (MES) per prior set, along with its 95%

confidence interval. The statistical significance of any demographic change is assessed by evaluating the 95% confidence intervals associated with the MES; non-significance is implied when the 95% confidence interval includes zero (Nakagawa and Cuthill 2007). Significant negative values suggest significant bottlenecks, while significant positive values signify population expansions.

CHAPTER 4

RESULTS

4.1 Survey Results

A total of 26 sites were surveyed for the presence of threatened freshwater mussel species and 9 produced sufficient numbers of *Q. aurea* for population genetic analysis. *Q. aurea* was found at 19 sites while *Q. petrina* and *F. mitchelli* were found at 9 and 4 sites respectively. Sampling numbers by site are detailed in Appendix B and totaled 549 for *Q. aurea* (range = 0 - 74), 142 for *Q. petrina* (range 0-51), and 23 for *F. mitchelli* (range 0-13) (Table 4.1). *Q. aurea* was found in all three river systems (Guadalupe, San Marcos, and San Antonio) while *Q. petrina* was found only in the Guadalupe and San Marcos systems and *F. mitchelli* was found only in the Guadalupe system.

Table 4.1. Sample results by river for three threatened freshwater mussel species in central Texas.

		Guadalupe	San Marcos	San Antonio	KW[1] *p* value
Quadrula aurea	Total	270	81	198	
	Mean Per Site	38.6	20.3	24.8	
	Mean Encounter Rate[2]	23.5	16.3	22.3	0.6259
Quadrula petrina	Total	60	82	0	
	Mean Per Site	12	20.5		
	Mean Encounter Rate[2]	8.7	14.7		0.7122
Fusconaia mitchelli	Total	23	0	0	
	Mean Per Site	5.8			
	Mean Encounter Rate[2]	2.9			NA

[1]Kruskal-Wallis test

[2]Encounter rate defined as numbers of mussels per person-hour.

Sampling results (total numbers, average numbers per site, and average encounter rates) were highest for *Q. aurea* in the Guadalupe River and highest for *Q. petrina* in the San Marcos River. Overall encounters per person-hour for *Q. aurea* and *Q. petrina* were both highest at the site above Luling on the San Marcos River, which is a small dense mussel bed. However, mean rank values for search efficiencies were not significantly different across rivers for either *Q. aurea* (Kruskal Wallace test, $\chi^2 = 0.9371$, $p = 0.6259$), or *Q. petrina* (Kruskal Wallace test, $\chi^2 = 0.1361$, $p = 0.7122$) which suggests that mussel beds of sufficient size to allow for the collection of genetic material tend to have similar densities across river systems.

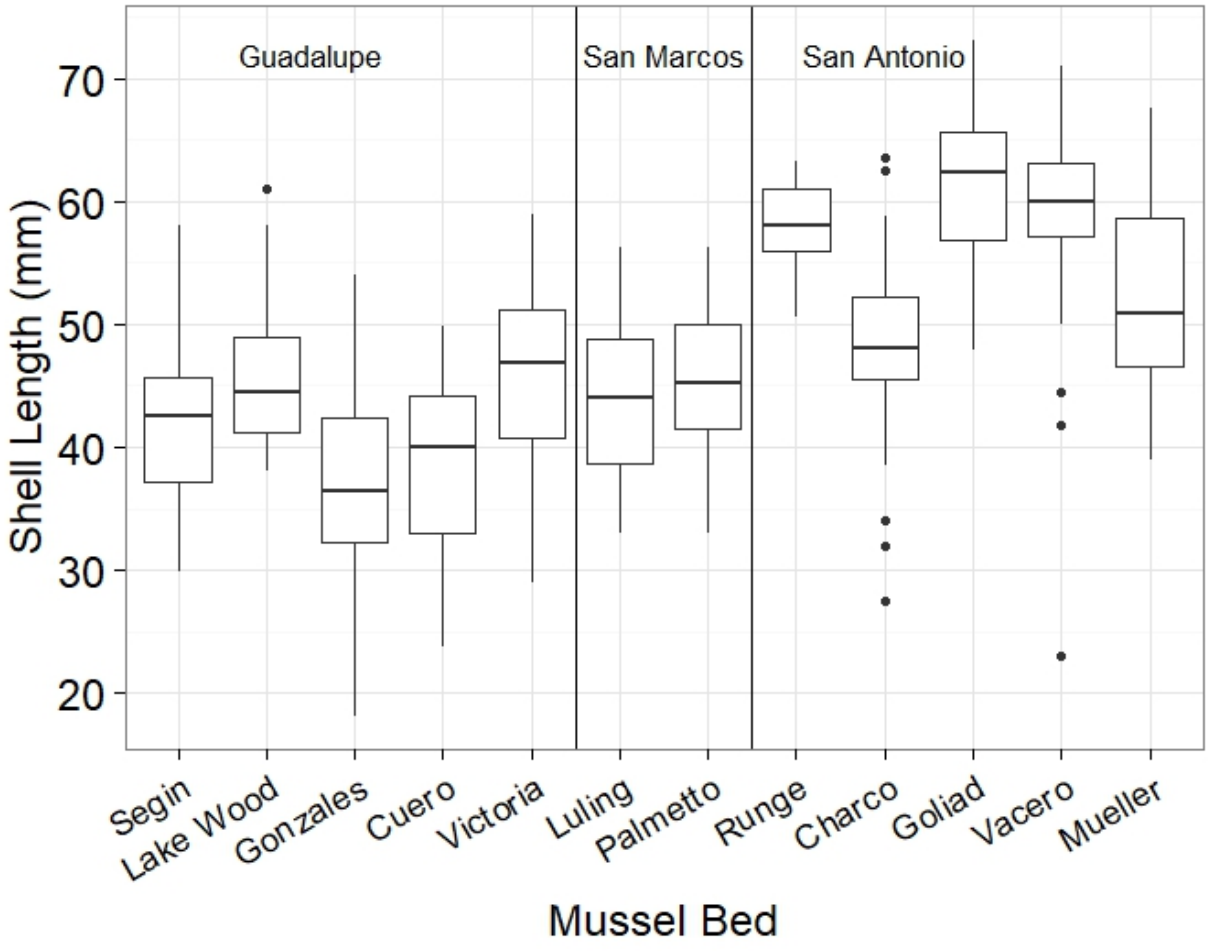

Figure 4.1. Shell length by sites and river for 12 populations of *Quadrula aurea* in the lower Guadalupe, San Marcos, and San Antonio rivers

Size distribution was determined for *Q. aurea* at all sites where genetic samples were collected as well as two other sites (Segin and Vacero) that produced sufficient numbers of mussels, but were not sampled for genetics. A twelfth site (Mueller Ranch on the San Antonio River) which was surveyed as part of a previous study by the author was also included in the size analysis to provide more information on San Antonio River populations. Median size tended to be smaller in the Guadalupe River and was smallest at Gonzales while sites in the San Antonio River tended to have larger (and presumably older) individuals. A Kruskel-Wallis test found length distributions to be significantly different among rivers ($\chi^2 = 240.9$, $p < 0.0001$). A Dunn's multiple comparison test indicated mussel length in both the Guadalupe and San Marcos rivers differed from the San Antonio. This result reflects the box plots created for shell length (Figure 4.1) which suggest median mussel lengths in populations in the San Antonio River are significantly longer than mussels from the Guadalupe and San Marcos Rivers.

One unexpected result from the survey effort was the discovery of two small populations of *Fusconaia mitchelli*, a species once thought to be extinct (Howells et al., 1996; Haag, 2009a; Howells, 2010). In the fall of 2012, six live specimens of *F. mitchelli* were discovered during a survey on the Guadalupe River near Cuero, Texas (Figure 4.2). The recently dead shells of an additional six specimens were also found at the same location. Another 13 live specimens were discovered in August of 2013 near Gonzalez, Texas. Field identification of *F. mitchelli* was verified by Robert Howells - a 22 year veteran of the Texas Parks and Wildlife Department and a recognized expert on freshwater mussels in Texas - using the recently dead shells. Both live and dead specimens represented a range of size classes; shell lengths for the live specimens were 31.5 - 57.5 mm while lengths of the recently dead were 23.8 - 48.0 mm.

Because shell length is an indicator of relative age in freshwater bivalves (Harmon and Joy, 1990, Haag, 2009b) this finding constitutes the first documentation of a reproducing population of *F. mitchelli* in at least 30 years (Howells, 2010).

Figure 4.2. Six live specimens of *Fusconaia mitchelli* from the Guadalupe River near Cuero, TX. These specimens represent the first documented occurrence of a reproducing population of *F. mitchelli* in over 30 years.

4.2 Habitat Measurements

Habitat parameters were measured at 11 locations where large mussel beds were found - 9 sites where genetic samples were collected and 2 genetic sampled were not collected. Information from the Mueller Ranch site on the San Antonio River, surveyed in 2011 by the author and colleagues for another study, was added to the habitat data set for the San Antonio

River. The complete set of habitat measurements are provided in several tables in Appendix C while values for the median and range per basin are given in Table 4.2. STATSGO2 soil data categories were not consistent across sampling locations and therefore the soil data presented in Table 4.2 represents the soil categories that were the most consistent across sites and constituted the highest percentage of overall soil composition.

Table 4.2. Median and range () by river for habitat variables measured at mussel sampling locations and calculated through GIS analysis (mm, millimeter; m, meter; km, kilometer; s, second; % percent).

		Guadalupe	San Marcos	San Antonio
Geomorphology (m)	Mussel Bed Width	27.5 (8.0-42.0)	16.6 (13-20.2)	5 (2.6-10)
	Wetted Width	34 (14.0-42.0)	22 (20.2-23.8)	19.5 (16.5-24.2)
	Bankfull Width	35.4 (29.5-44.0)	23.4 (20.2-26.5)	23.8 (19.6-31)
	Ave Depth	0.53 (0.27-0.74)	0.48 (0.46-0.50)	0.64 (0.40-1.04)
	Avg Bankfull Depth	1.24 (0.80-1.4)	0.93 (0.86-1.00)	1.23 (0.93-1.94)
	Gradient (m/km)	0.25 (0.13-1.91)	0.57 (0.35-0.79)	0.18 (0.14-0.53)
Streamflow (m/s)	Ave Velocity 60% Depth	0.56 (-0.01-0.79)	0.35 (0.26-0.44)	0.22 (0.05-0.35)
	Avg Velocity Near Bed	0.40 (-0.01-0.45)	0.20 (0.15-0.26)	0.1 (0.02-0.22)
Sediment Size (mm)	D16	1.03 (0.06-7.29)	0.46 (0.30-0.63)	0.3 (0.05-0.40)
	D50	21.34 (0.19-24.59)	17.45 (10.79-24.10)	11.31 (0.10-22.43)
	D84	46.32 (0.41-108.00)	43 (36.35-49.66)	45.69 (0.22-49.71)
	Geometric mean	11.46 (0.18-15.26)	7.34 (6.37-8.30)	4.49 (0.11-8.16)
Complex Hydrologic Variables	RSS	0.129 (0.006-0.252)	0.038 (0.033-0.043)	0.022 (0.004-0.066)
	Re	7229 (1.1-26589)	3574 (2016-5132)	1610 (0.8-4166)
	F	0.202 (0.007-0.227)	0.094 (0.069-0.119)	0.031 (0.015-0.111)
Landuse (%)	Developed	0.07 (0.03-0.29)	0.13 (0.10-0.16)	0.06 (0.04-0.27)
	Forest	0.10 (0.08-0.16)	0.22 (0.14-0.29)	0.1 (0.04-0.24)
	Agriculture	0.56 (0.29-0.63)	0.29 (0.28-0.31)	0.33 (0.29-0.59)
	Shrubland	0.15 (0.12-0.27)	0.28 (0.25-0.32)	0.28 (0.24-0.29)
	Herbaceous	0.02 (0.02-0.03)	0.02 (0.02-0.03)	0.03 (0.02-0.06)

(table continues)

		Guadalupe	San Marcos	San Antonio
Soil Composition %	s7265	12.4 (0.0-45.6)	61.6 (50.3-73.0)	0.0 (0.0-0.0)
	s7462	39.0 (0.0-53.6)	20.0 (14.2-25.8)	0.0 (0.0-0.0)
	S7718	0.0 (0.0-5.5)	0.0 (0.0-0.0)	37.4 (0.0-38.7)
	S7719	0.0 (0.0-0.0)	0.0 (0.0-0.0)	18.0 (9.8-39.9)
	S9710	0.0 (0.0-0.0)	0.0 (0.0-0.0)	26.6 (22.8-29.5)

4.2.1 Habitat Measurements: Statistical Relations among River Basins

Statistical tests for differences among rivers were generally not significant with the exception of mussel bed width, bankfull width, and the soil composition variables (Table 4.3). A Kruskal-Wallis test found the distribution of mussel bed widths to be significantly different among rivers (χ^2=7.3385, p = 0.0255) and a subsequent Dunn's multiple comparison test found a difference between Guadalupe and San Antonio, but no difference between the Guadalupe and San Marcos or San Marcos and San Antonio (Figure 4.3). The Wilcoxon test also found a statistically significant difference between mussel bed widths in the Guadalupe/San Marcos and San Antonio under the two-basin model (Wilcoxon =1, p = 0.0051). Bankfull widths at mussel bed sites were found to be significantly different among rivers (χ^2=7.2692 p=0.02639). The Dunn's multiple comparison test could not separate basins at the α = 0.05 level, but the Guadalupe differed from the San Marcos and the San Antonio at p = 0.055 and 0.054 respectively. While the two-basin model was not significant for bankfull width, a graphic comparison indicates sites in the Guadalupe River are wider then sites in the San Antonio or San Marcos (Figure 4.4). All soil composition variables were highly statistically significant under both models. The soil results reflect the basin specific soil composition and the fact that soil

types that appear in the Guadalupe/San Marcos basin are absent from the San Antonio and vice versa (Table 4.2).

Median values for some habitat variables appear to be different among rivers, but large data variances likely make differences in distribution non-significant under the relatively low power non-parametric tests used in this analysis. For example, the median value for the Geometric mean of sediment particle size values for the Guadalupe River is 87% larger than the San Antonio River, but neither the Kruskel-Wallis test under the three-basin model or the Wilcoxon test under the two basin model were able to statistically separate the Guadalupe River likely due to the large variance in values for the Guadalupe river (Table 4.2). With specific reference to the Geometric mean example the large variance is related to a single sample taken in association with a mussel bed found in fine sediment along the river's edge.

Table 4.3. Results of statistical tests for habitat differences under the three-basin model (Kruskel-Wallis rank sum test) and the two-basin model (Wilcoxon rank sum test).

	Three Basins (Guadalupe, San Marcos, and San Antonio)		Two Basins (Guadalupe/San Marcos and San Antonio)	
Measurement	Kruskal-Wallis	*p*	Wilcoxon	*p*
Mussel Bed Width	7.3385	0.0255	1	0.0051
Wetted Width	3.1231	0.2098	8	0.149
BankFull Width	7.2692	0.02639	7	0.1061
Avergae Depth	1.1846	0.553	24	0.3434
Avergae Bankfull Depth	1.3615	0.5062	24	0.3434
Gradient	2.2923	0.3179	13	0.5303
Avg Velocity 60 % Depth	1.4846	0.476	10	0.2677
Aveg Velocity Near Bed	2.3846	0.3035	8	0.149
D16	3.3923	0.1834	7	0.1061
				(table continues)
D50	1.4846	0.476	10	0.2677

	Three Basins (Guadalupe, San Marcos, and San Antonio)		Two Basins (Guadalupe/San Marcos and San Antonio)	
D84	0.3231	0.8508	14	0.6389
Geometric Mean	3.5769	0.1672	6	0.07323
RSS	0.934	0.6269	12	0.416
Re	2.4077	0.3	8	0.149
F	1.9154	0.3838	9	0.202
Developed	1.2769	0.5281	13	0.5303
Forest	2.2923	0.3179	15	0.7551
Agriculture	3.0395	0.2188	17.5	1
Shrubland	5.5714	0.06169	27.5	0.1222
Herbaceous	2.5123	0.2847	27	0.1396
s7265	7.8913	0.01934	30	0.02977
s7462	7.0821	0.02898	32.5	0.01195
s7718	6.0643	0.04821	4	0.01856
s7719	10.043	0.00659	0	0.00208
s9710	10.043	0.00659	0	0.00208

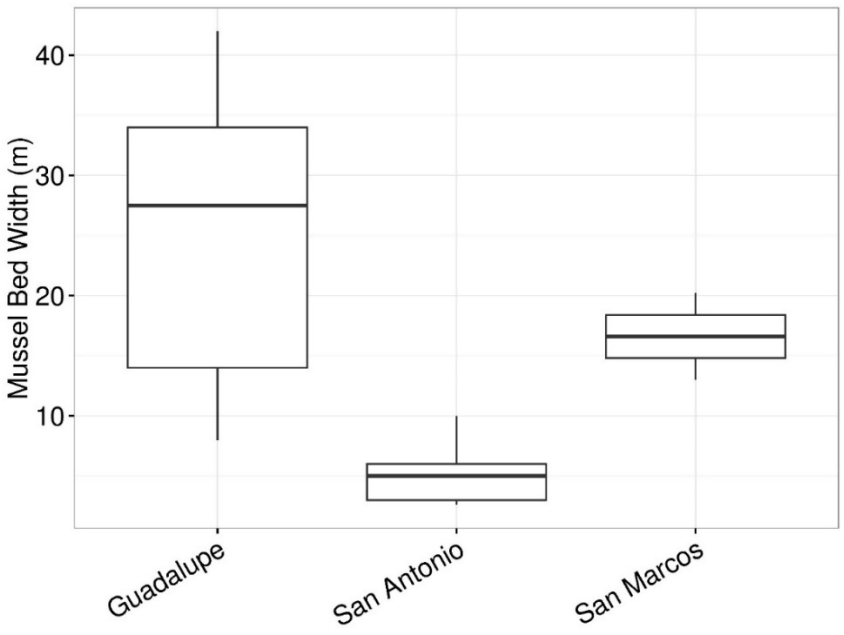

Figure 4.3. Distribution of mussel bed width measurements by river. Plots describe the median, 25th quantile, 75th quantile, minimum, and maximum vales, and any outliers.

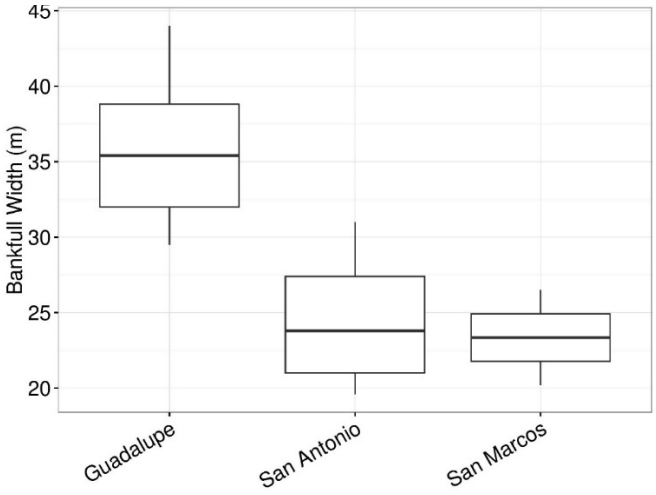

Figure 4.4. Distribution of bankful width measurements by river. Plots describe the median, 25th quantile, 75th quantile, minimum, and maximum vales, and any outliers.

4.2.2 Habitat Measurements: Spearman Correlation

The results of the Spearman correlation analysis among habitat variables is presented in Figure 4.5. Many correlation values among categorically related variables were found to be relatively large and could be either positive or negative. For example, the sediment particle size variables D84, D50, and the Geometric mean (GeoM) were highly positively correlated. In contrast, some of the soil composition variables tended to be highly negatively correlated - likely due the fact that many soils types were present in one basin and absent in the other. The percentage of land cover as agriculture (Agricul) was negatively correlated with the percentage of land cover as Forest or Shrubland. Other correlations appeared to be related to the fundamental differences between river basins. Mussel bed width (BW), which differs significantly between river basins (Figure 4.3) was also correlated with several other variables that differ between the Guadalupe/San Marcos and San Antonio basins: sediment composition variables, wetted width (WW), and some flow variables. Another common pattern in the data is

the high correlation among variables that influence other variables. The calculation of the complex flow variables such RSS, Reynolds number (Re), and Froud number (Fr) is dependent on flow velocity and sediment composition. As a consequence, these variables tended to be relatively highly correlated.

Few variables were uncorrelated or only weakly correlated with other variables. The sediment variable D16 showed only a moderately high correlation with the related sediment variable GeoM and with the percentage of land cover as developed land (Devlp). Devlp itself was not highly correlated with any variables.

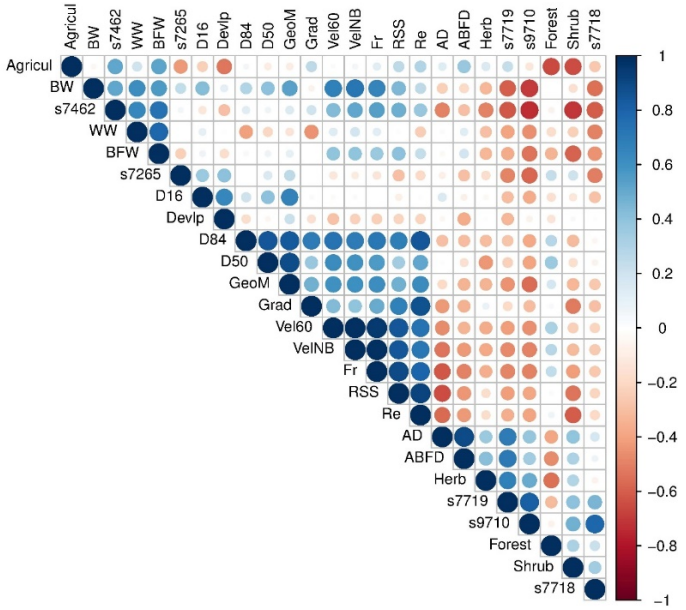

Figure 4.5. Spearman correlation matrix of habitat variables measured at *Quadrula aurea* sampling locations.

4.2.3 Habitat Measurements: Principle Components Analysis

A principle components analysis was used, in association with the Spearman correlation analysis, to analyze the relations between habitat variables and to select a set of variables that 1) represent the suit of habitat components measured as part of the habitat analysis and 2) would be used to develop an estimate of environmental distance between sampling sites to be

analyzed against genetic distance. The loadings for the first three principle components are presented in Table 4.4. The variables with the highest loadings for the first three principle components were selected for the development of environmental distance matrix. While mussel bed width (BW) had the highest loading in the first principle component (PCA1) under the Stream/Bed Size group, wetted width, which had the highest loading under PCA2, was selected due to the relatively high correlation between BW and some of the other habitat variables (Figure 4.5). While a single variable was selected for each variable group to reduce the potential for variable correlation, several soil variables were selected to incorporate the dichotomy between river basins.

Table 4.4. Results of the principle components analysis with the full suite of habitat variables. Variables in bold were selected for the development of the environmental distance matrix.

Variable Group	Variable	PC1	PC2	PC3
Stream/Bed Size	BW	0.223	0.143	0.197
	WW	0.069	**0.412**	0.181
	BFW	0.157	0.387	-0.032
Stream Depth	**AD**	**-0.207**	0.082	-0.026
	ABFD	-0.179	0.195	-0.156
Flow Velocity	Vel60	0.28	-0.067	-0.105
	VelNB	**0.289**	-0.044	-0.077
	Grad	0.185	-0.146	-0.222
Sediment Size	D16	0.063	-0.036	0.293
	D50	0.198	-0.213	0.015
	D84	**0.239**	**-0.245**	-0.138
	GeoM	0.235	-0.177	0.089
Landuse	Devlp	-0.029	-0.096	0.344
	Forest	0.071	-0.299	0.133
	Agricul	0.045	0.321	-0.323
	Shrub	-0.193	-0.196	0.123
	Herb	**-0.166**	0.013	-0.175
Complex Hydrologic Variables	RSS	0.273	-0.025	-0.216
	Re	0.265	-0.111	-0.233
	Fr	0.298	-0.049	-0.11

(table continues)

Variable Group	Variable	PC1	PC2	PC3
Soil Composition	s7265	0.033	-0.066	0.403
	s7462	**0.221**	**0.315**	0.008
	s7718	-0.15	**-0.254**	-0.154
	s7719	**-0.234**	-0.039	-0.296
	s9710	**-0.247**	-0.156	-0.224

A second principle components analysis was performed using only the variables selected for calculating environmental distance among mussel sampling sites (Figure 4.6). Eigenvalues for the first two PCAs accounted for 70% of the variation between sampling sites.

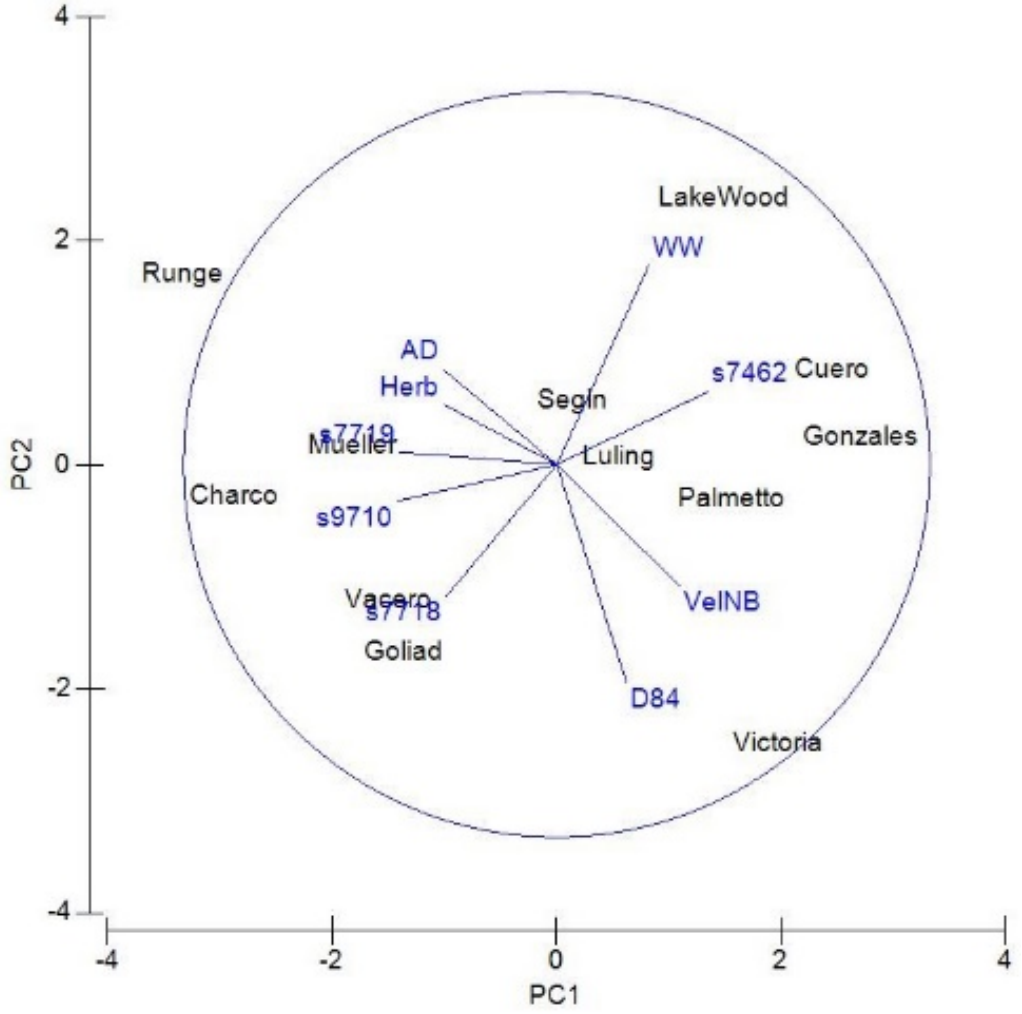

Figure 4.6. Visualization of the principle components analysis using only the variables selected for calculating environmental distance among mussel sampling sites.

4.3 Marker Identification

Thirteen of the 27 loci consistently amplified *Q. aurea* DNA and six were polymorphic (Table 4.5). The remaining 14 loci either did not amplify target DNA or were unresolved due to multiple PCR products. No loci originally developed for *Q. pustulosa* amplified in *Q. aurea*.

Table 4.5. Twenty-seven microsatellite loci screened for polymorphism in *Quadrula aurea*; *n*, sample size; T_A, annealing temperature; Hem, Hemmingsen et al. 2009; Roe, Kevin Roe pers. comm.; F, forward primer; R; reverse primer.

Locus and Sample Size	GenBank Accession no.	Source	Repeat Motif	T_A °C	Primer Sequence (5'-3')
Polymorphic Loci					
QfA130 *n* = 34	FJ785631	Hem	(TG)	58	F:TGAGAAATCGTGATGACTCAG R:CCTACCTACCTTCATGTGGTC
QfC6 *n* = 34	NA	Roe	(TACA)	58	F:CCACATTACACACACATACACG R:CGCCTCAAGACCTCTGAC
QfC109 *n* = 34	FJ785633	Hem	(TATG)	55	F:GACAGGAAATAAAGGGTGTC R:GCAATGTAATATGGTATGCAC
QfC114 *n* = 34	FJ785634	Hem	(TACA)	58	F:TCCATGTTTTTCTCCTCCTCTA R:CACCCTTGCTTATAGCGTAGTC
QfD5 *n* = 34	NA	Roe	(TG)	56	F:CGCAATATCAGACACAGTGG R:CAACCATAGTCACACTTGAGCA
QfD103 *n* = 34	FJ785641	Hem	(ATCT)	55	F:ACGTGTAACCGATTGGTATATC R:GTATGAAGGGACGAAAATGTAC
Monomorphic Loci					
Qf1A *n* = 15	FJ785638	Hem	(GTT)	55	F:ACAGTTCTAGTGTCCGAGGAGTCACTGG R:GGTGTATTGTGTCATCGGTGCTGCCA
QfA103 *n* = 10	FJ785629	Hem	(CA)	49	F:GCACACCTTATTCATTTGAGA R:AATGTCTTCCCCATGACTAAA
QfA112 *n* = 16	FJ785630	Hem	(CA)	56	F:ACTTGCTCCAAAACTTGTAGAG R:GGAATGGTTCAGACTATGACC
QfH8 *n* = 10	FJ785637	Hem	(AAC)	55	F:ACCCTTGTGGGTGTGGTGTGGAGAACG R:GGATCCAATCGGAGAGCCTGAGGT
QfN11 *n* = 10	FJ785642	Hem	(AC)	58	F:TGTGGCTGTGCTGGTGACTCATTTCC R:CCATGCCATCAGGTGCAGGA
QfO2 *n* = 10	FJ785644	Hem	(TG)	57	F:AGCAGACTTCATCGAGACAAAAATGGTCGG R:CCAGTTCATCAGTCGGTATATTCTTCCGCT
QfR9 *n* = 10	FJ785639	Hem	(CA)	66	F:AGCTTGGGATCGGAGTTGCAGCCAGC R:GGACACCCCAGTGTGTAAGAACA

(table continues)

Locus and Sample Size	GenBank Accession no.	Source	Repeat Motif	T_A °C	Primer Sequence (5'-3')
Unresolved or No Amplification					
QfC4 *n* = 4	FJ785632	Hem	(TACA)	NA	F:TGTCCTTCTCTGTGAATGTTTG R:GCACTCCATAAATGCAGGTAAT
QfC2 *n* = 10	NA	Roe	Unknown	NA	F:CCATTATTGGCGGGACAG R:AACCCACGCAGACAGAGG
QfC12 *n* = 11	NA	Roe	Unknown	NA	F:GACGGACAGATGAAATAGATGC R:CTTTTGTTGCGATGTTAGTCG
QfC102 *n* = 22	NA	Roe	Unknown	NA	F:TGCTTTCCTTTATCCCAAATC R:ATGGCAGATAGGTCAGTCATG
QfD2 *n* = 24	FJ785640	Hem	(ATAG)	NA	F:TGGATGTTATTGTGCTTAACGA R:GCCATTTATCAAAGAATGCAG
QfD102 *n* = 4	FJ785635	Hem	(ATCT)	NA	F:TGGACAATTCATCAAGTCAAG R:CTTTGTTTTCCAAACCATACAG
QFD110 *n* = 11	NA	Roe	Unknown	NA	F:TCTGCTGGAACTAGACAGGTG R:CGGATAAGAAAGAAGGGACAG
QfD116 *n* = 24	FJ785642	Hem	(TAGA)	NA	F:CCATGTAAAGGTTTGCATTAAC R:TGGACACACCACATATACAGAC
QfN9 *n* = 18	FJ785636	Hem	(TG)	NA	F:TCGTCTACCACCTCTGCAACACATACCG R:GGCAGAGAGGTCACAACCCCGGA
QfP5 *n* = 24	FJ785645	Hem	(CAC)	NA	F:TCGCCACGGTACAATCAGTTCTTGCAACG R:GCGTGTCTGACGAGCAATAGGT
Unresolved or No Amplification					
PcC6 *n* = 8	NA	Roe	Unknown	NA	F:GCAGTGTATGGCAATGAACA R:GCGTAATAACCTGTGACCTCC
PcC105 *n* = 8	NA	Roe	Unknown	NA	F:TTGCATGTGTCACTTCATACTG R:GCACCTACCTACCTATCTCTCG
PcC125 *n* = 8	NA	Roe	Unknown	NA	F:GGACGCTCTAACCACTAGGC R:AGTCCAGATTTGATTGCTTCAG
PcD113 *n* = 8	NA	Roe	Unknown	NA	F:TAAAAGAAGCTCCATCACATG R:AGTTGCATCAGTTGTATGATTG

Variation was relatively high among the six polymorphic loci in *Q. aurea*; we genotyped 34 individuals from a single mussel bed and found total number of alleles by loci ranged from six to 17 (mean ± s.d. = 11.8 ± 3.9, Table 4.3). Expected heterozygosity (H_E) by loci ranged from 0.531 to 0.948 (0.798 ± 0.160). No significant deviations from Hardy-Weinberg equilibrium were detected which suggests our samples were collected from a single interbreeding population

(i.e., there is no evidence to suggest hidden population subdivision due to sampling multiple populations). Tests for linkage disequilibrium were not significant after correction for multiple comparisons, which indicates statistical independence among alleles at different loci. Screening with the MICRO-CHECKER program found no evidence for genotyping errors.

Table 4.6. Characteristics of 6 loci that tested as polymorphic in the freshwater mussel *Quadrula aurea*; bp = base pair; H_O = observed heterozygosity; H_E = expected heterozygosity.

Locus	Size Range (bp)	Median Size (bp)	No. Alleles	H_O	H_E
QfA130	231 - 275	241	17	0.882	0.885
QfC6	169 - 262	218	16	0.824	0.921
QfC109	153 - 187	153	6	0.471	0.447
QfC114	225 - 257	241	9	0.824	0.834
QfD5	156 - 196	168	13	0.765	0.875
QfD103	229 - 266	237	10	0.912	0.827

4.3.1 Loci Characteristics

The characteristics of loci that amplified within *Q. aurea* appeared similar to the source species, but with one exception (Table 4.7). Roe and Boyer (2015) reported a tetranucleotide or four-step repeat sequence of ATCT in the QfD5 locus within *Q. fragosa* and *Q. pustulosa*. Results of this analysis indicate that variation among QfD5 alleles in *Q. aurea* reflects a dinucleotide (two-step) repeat sequence.

Table 4.7. Comparison of microsatellite loci characteristics among *Quadrula aurea* and the two microsatellite source species, *Q. fragosa*, and *Q. pustulosa*. NR = not reported; NA = not applicable.

Species	*Quadrula aurea*			*Quadrula fragosa*[†]			*Quadrula pustulosa*[††]		
	Sample Size	No. Alleles	Repeat Motif	Sample Size	No. Alleles	Repeat Motif	Sample Size	No. Alleles	Repeat Motif
QfA130	34	17	TG	53	12	TG	39	23	TG
QfC6	34	16	TACA	53	14	TACA	39	22	TACA
QfC109	34	6	TATG	53	9	TATG	39	6	TATG

(table continues)

Species	*Quadrula aurea*			*Quadrula fragosa*†			*Quadrula pustulosa*††		
	Sample Size	No. Alleles	Repeat Motif	Sample Size	No. Alleles	Repeat Motif	Sample Size	No. Alleles	Repeat Motif
QfC114	34	9	TACA	43	7	TACA	(NA)	(NA)	(NA)
QfD5	34	13	TG	53	7	ATCT*	39	9	ATCT*
QfD103	34	10	ATCT	52	2	NR	(NA)	(NA)	(NA)

† Diversity measures for Q. fragosa were obtained from Roe and Boyer (2015), Roe (2010), and Hemmingsen et al. (2009). †† Diversity measures for Q. pustulosa were obtained from Roe and Boyer (2015). * The complementary sequence (TAGA) is reported in Roe and Boyer (2015)

Because the potential exists for primers to amplify non-orthologous products in closely related species (Yue et al. 2010), we compared a 169 bp region of the QfD5 locus derived from *Q. fragosa* (provided by Dr. Kevin Roe) to seven QfD5 sequences obtained for *Q. aurea* (Appendix E). Flanking regions surrounding this locus were similar among all individuals, indicating the QfD5 locus is orthologous between *Q. aurea* and *Q. fragosa*. However, the microsatellite region differed considerably between species; both species contained composite microsatellites with the same tetranucleotide sequence (ATCT) but with different dinucleotide sequences (CT in *Q. fragosa* and GT in *Q. aurea*). Moreover, the tetranucleotide sequence was fixed at three repeats in *Q. aurea* and variation was only associated with the dinucleotide sequence. A third repetitive array that differed slightly in sequence between species, but appeared to be fixed in length in *Q. aurea*, preceded the variable microsatellite region in both species and contained an $(AC)_4$ repeat in *Q. aurea* and an $(AC)AT(AC)_2$ in *Q. fragosa*.

All loci except QfC114 revealed inconsistencies in scoring patterns by parity switching (changing from odd to even) at least once in allele scoring. Most parity changes involved a single base-pair increase in the size between two alleles (e.g., a 5-step change in a tetranucleotide repeat from 235 to 240) and appeared to involve insertion/deletion events in the microsatellite flanking regions. For example, two of the three samples we sequenced at the

QfC109 locus demonstrated a change in parity that was associated with a single base-pair insertion in the 3' flanking region. Sequencing of the QfD5 and QfC109 loci also revealed alleles with one or more point mutations (single nucleotide polymorphisms or (SNPs) in their flanking regions. One instance of size homoplasy was found in the QfD5 locus (Table D1). Five of the six alleles sequenced for QfD5 and scored at 156 bp contained adenine at position 147 (relative to the *Q. fragosa* sequence) while one was scored as 156, but contained guanine at the 147 position.

4.3.2 Power Analysis

The power analysis using the POWSIM program showed the overall study design has high statistical power to identify genetic divergence among *Q. aurea* populations at the F_{ST} = 0.01 level. The proportion of significant outcomes ($p \leq 0.05$) under the Fisher's exact test, with a model defined true F_{ST} value of 0.01, was 100%. The probability for type I error (i.e., rejecting the null hypothesis of no difference among populations when it is in fact true) was calculated as 4.8% (i.e., $p = 0.048$).

4.4 *Q. aurea* Genetic Structure

4.4.1 Neutral Genetic Diversity

All loci were highly polymorphic with a total of 110 alleles and a mean of 18.3 unique alleles/loci (Table 4.8). Private alleles were identified in 5 of the 6 loci with the highest number (5) found in the uppermost site on the Guadalupe River (Lake Wood) above the confluence of the San Marcos River. The two sites that had no private alleles were Palmetto on the San

Marcos River and Gonzales just below the confluence of the San Marcos and Guadalupe rivers. We found no significant probability for genotyping errors in any of the 6 loci and no significant deviations from Hardy-Weinberg expectations or linkage disequilibrium after correction for multiple comparisons.

Within-population genetic diversity was similar among all populations (Table 4.5). Mean A_R values ranged from 10.65 to 12.10 (S.E. = 0.42) alleles per locus and mean H_E values ranged from 0.78 to 0.84 (S.E. = 0.02). Kruskal-Wallace tests found no significant differences among populations in A_R ($\chi^2 = 0.70$, $p = 0.9995$) or H_E ($\chi^2 = 0.78$, $p = 0.9993$). The lowest mean values for both A_R and H_E were found in the San Antonio River (Goliad and Runge respectively) and the largest values were in the Guadalupe River (Lake Wood and Gonzales respectively). Multilocus estimates of the inbreeding coefficient F_{IS} ranged from -0.058 to 0.075 and suggest little reduction in heterozygosity relative to a randomly mating population. F_{IS} displayed more variation among populations, but differences were again not significant ($\chi^2 = 13.63$, $p = 0.0921$). The largest F_{IS} value was found at the uppermost site sampled in the San Marcos River (Luling), while the smallest value was found at Gonzales below the confluence of the Guadalupe and San Marcos rivers. Both Gonzales and Victoria had slightly negative estimates of F_{IS} indicating these populations have a slight heterozygote excess relative to Hardy-Weinberg expectations.

Table 4.8. Characteristics of 6 microsatellite loci genotyped within 9 spatially discrete populations of the freshwater mussel *Quadrula aurea*. Private alleles in parenthesis *()*; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; *n*, sample size; bp, base pairs. Site names are abbreviated as Ru, Runge; Ch, Charco; Go, Goliad; Vi, Victoria; Cu, Cuero; Gz, Gonzales; LW, Lake Wood; Pa Palmetto; Lu, Luling.

Locus names and Allele size range (bp)		San Antonio			Guadalupe				San Marcos		Total # Unique Alleles
		Ru	Ch	Go	Vi	Cu	Gz	LW	Pa	Lu	
QfA130 231-275	# of alleles	16*(1)*	16*(1)*	17*(1)*	16*(0)*	15*(0)*	15*(0)*	14*(0)*	14*(0)*	16*(0)*	21*(3)*
	H_O	0.86	0.93	0.88	1.00	0.86	0.93	0.87	0.89	0.87	
	H_E	0.94	0.93	0.88	0.91	0.91	0.91	0.85	0.88	0.92	
	n	22	27	34	31	28	29	30	27	30	
QfC109 145-196	# of alleles	4*(0)*	6*(0)*	6*(0)*	8*(1)*	6*(0)*	9*(0)*	10*(1)*	9*(0)*	8*(0)*	12*(2)*
	H_O	0.23	0.30	0.47	0.58	0.36	0.62	0.47	0.48	0.47	
	H_E	0.25	0.27	0.45	0.50	0.43	0.60	0.58	0.54	0.57	
	n	22	27	34	31	28	29	30	27	30	
QfD103 225-294	# of alleles	9*(0)*	11*(1)*	10*(0)*	11*(2)*	9*(1)*	10*(0)*	10*(1)*	10*(0)*	10*(0)*	16*(5)*
	H_O	0.77	0.89	0.91	0.94	0.86	0.90	0.80	0.89	0.72	
	H_E	0.80	0.84	0.83	0.88	0.85	0.88	0.88	0.87	0.84	
	n	22	27	34	31	28	29	30	27	29	
QfC6 153-262	# of alleles	19*(0)*	17*(2)*	16*(1)*	17*(0)*	22*(0)*	19*(0)*	22*(2)*	21*(0)*	17*(1)*	30*(6)*
	H_O	0.96	0.93	0.82	0.97	0.96	1.00	0.97	0.96	0.93	
	H_E	0.94	0.93	0.92	0.93	0.95	0.93	0.95	0.95	0.93	
	n	22	27	34	31	28	29	30	26	29	
QfC114 225-269	# of alleles	9*(0)*	8*(0)*	9*(0)*	9*(0)*	7*(0)*	9*(0)*	11*(1)*	8*(0)*	8*(0)*	11*(1)*
	H_O	1.00	0.78	0.82	0.77	0.68	0.90	0.87	0.85	0.79	
	H_E	0.86	0.83	0.83	0.83	0.75	0.82	0.84	0.81	0.82	
	n	22	27	34	31	28	29	30	27	29	

(table continues)

Locus names and Allele size range (bp)		San Antonio			Guadalupe				San Marcos		Total # Unique Alleles
		Ru	Ch	Go	Vi	Cu	Gz	LW	Pa	Lu	
QfD5 156-198	# of alleles	13*(0)*	18*(0)*	13*(0)*	13*(0)*	14*(0)*	14*(0)*	13*(0)*	13*(0)*	13*(0)*	20*(0)*
	H_O	0.86	0.89	0.76	0.84	0.82	0.97	0.77	0.74	0.83	
	H_E	0.91	0.93	0.88	0.87	0.90	0.89	0.84	0.85	0.90	
	n	22	27	34	31	28	29	30	27	30	
Mean Alleles per locus		11.67	12.67	11.83	12.33	12.17	12.67	13.33	12.50	12.00	
Mean Allelic richness		11.67	11.85	10.65	11.25	11.23	11.78	12.10	11.63	11.15	
Mean H_E by population		0.78	0.79	0.80	0.82	0.80	0.84	0.82	0.82	0.83	
Mean F_{IS} by population		0.013	0.006	0.024	-0.035	0.051	-0.058	0.042	0.018	0.075	

4.4.2 Genetic Structure

Weak, but significant genetic structure was evident between the San Antonio and Guadalupe/San Marcos drainage basins, but not among populations within drainage basins. The AMOVA analysis indicates the majority of genetic variation resides within populations, but variance between drainage basins accounted for 1.69 % of total genetic variation (Table 4.9, Panel A). A locus-by-locus AMOVA was used to determine if genetic structure results were consistent throughout the data set. Levels of variation within populations (F_{ST}) and among drainages (F_{CT}) were significant across all loci with the exception of QfC114 (Table 4.9, Panel B). Estimates for F_{ST} and F_{CT} calculated by loci were generally larger than the values calculated using the combined data set. A lack of variation in the QfC114 locus likely reduced the overall estimate of variation accounted for by structure between drainages. Within drainage variation (F_{SC}) by loci was not dramatically different from the overall estimate and a significant result was found only at locus QfA130 which displayed relatively weak variation.

Table 4.9. Panel A: Results of analysis of molecular variance (AMOVA) for the freshwater mussel *Quadrula aurea*; the Guadalupe and San Marcos rivers were combined into one drainage for this analysis. Panel B: Locus specific hierarchical *F*-statistics for *Q. aurea*.

Panel A

Source of Variation	d.f.	Sum of Squares	Percentage of Variation	*p* Value (SD)	*F*-Statistic
Among drainages	1	12.0	1.69	0.0118 (0.0004)	F_{CT} = 0.0170
Among populations within drainages	7	17.9	0.09	0.3009 (0.0015)	F_{SC} = 0.0010
Within populations	507	1227.1	98.21	<0.0000 (0.0000)	F_{ST} = 0.0179
Total	515	1256.9			

Panel B

Locus	Among Drainages (F_{CT})	Within Drainages (F_{SC})	Within Populations (F_{ST})
All	0.0170*	0.001	0.0179*
QfA130	0.0188*	0.0054*	0.0241*
AfC109	0.0259*	-0.0017	0.0242*
QfD103	0.0235*	-0.0001	0.0234*
QfC6	0.0139*	-0.0011	0.0128*
QfC114	0.0041	0.0039	0.008
QfD5	0.0184*	-0.0013	0.0171*

* Indicates significance at α = 0.05

Pair-wise results for F_{ST}, R_{ST}, (Table 4.10) and D_{est} (Table 4.11) were similar and reflected weak genetic structuring among drainage basins; significant differences, after correction for multiple comparisons, occurred primarily between populations separated by the confluence of the San Antonio and Guadalupe/San Marcos rivers. In contrast, pairwise estimates among populations within the same drainage were generally not significantly different. R_{ST} estimates and the Stepwise Mutation Model detected two significant pairwise differences between populations (Charco - Cuero, and Runge - Goliad) that were non-significant under F_{ST} and the Infinite Alleles Model. The D_{est} estimator was similar to F_{ST}, but identified the pairwise differences between Charco - Cuero, and Lake Wood - Gonzales as statistically significant.

The multilocus comparison between pR_{ST} and R_{ST} was strongly significant ($p < 0.0000$; Table 4.12) indicating that stepwise mutations contribute to genetic differentiation in these microsatellite markers. However, the locus specific analyses revealed only two of the six markers, QfC6 and QfD5, showed a significant difference between pR_{ST} and R_{ST}. These loci were hypervariable and among the most polymorphic used in this study.

Table 4.10. Pair-wise F_{ST} (lower triangle) and pairwise R_{ST} (upper triangle) among all *Quadrula aurea* populations. Values in bold were significant at $\alpha = 0.05$ after correction for multiple comparisons.

	San Antonio			Guadalupe				San Marcos	
	Runge	Charco	Goliad	Victoria	Cuero	Gonzales	Lake Wood	Palmetto	Luling
Runge	--	-0.0104	**0.0484**	**0.1423**	**0.1058**	**0.1012**	**0.1206**	**0.1250**	**0.1047**
Charco	-0.0039	--	0.0341	**0.1780**	**0.1440**	**0.1352**	**0.1514**	**0.1530**	**0.1410**
Goliad	0.0089	0.0015	--	**0.1091**	**0.0997**	**0.0801**	**0.0887**	**0.0861**	**0.0885**
Victoria	**0.0172**	**0.0079**	**0.0149**	--	-0.0082	-0.0112	-0.101	-0.0094	-0.0088
Cuero	**0.0170**	0.0095	**0.0147**	0.0003	--	-0.0088	-0.0013	-0.0061	-0.0147
Gonzales	**0.0232**	**0.0109**	**0.0143**	-0.0051	-0.0002	--	-0.0132	-0.0090	-0.0152
Lake Wood	**0.0319**	**0.0222**	**0.0314**	0.0019	0.0092	0.0076	--	-0.0125	-0.0127
Palmetto	**0.0250**	**0.0118**	**0.0181**	-0.0039	-0.0012	-0.0020	-0.0050	--	-0.0089
Luling	**0.0260**	**0.0133**	**0.0215**	-0.0013	0.0057	-0.0004	0.0006	-0.0020	--

Table 4.11. Pair-wise D_{est} estimates (lower triangle) and associated p values (upper triangle) among all *Quadrula aurea* populations. Significant results ($p \leq 0.05$) are in bold type for easy identification.

	San Antonio			Guadalupe				San Marcos	
	Runge	Charco	Goliad	Victoria	Cuero	Gonzales	Lake Wood	Palmetto	Luling
Runge	0	0.9567	0.1421	**0.0083**	**0.0060**	**0.0033**	**0.0033**	**0.0033**	**0.0033**
Charco	-0.0451	0	0.5266	**0.0180**	**0.0474**	**0.0148**	**0.0033**	**0.0120**	**0.0135**
Goliad	0.0401	0.0039	0	**0.0033**	**0.0033**	**0.0033**	**0.0033**	**0.0033**	**0.0033**
Victoria	**0.1119**	**0.0610**	**0.1126**	0	0.6729	0.9361	0.2453	0.6725	0.4575
Cuero	**0.1019**	**0.0608**	**0.1151**	-0.0105	0	0.5928	0.0792	0.7258	0.4196
Gonzales	**0.1422**	**0.0661**	**0.1096**	-0.0344	-0.0048	0	**0.0103**	0.5035	0.3863
Lake Wood	**0.1896**	**0.1387**	**0.1983**	0.0204	0.0496	**0.0661**	0	0.9690	0.4842
Palmetto	**0.1335**	**0.0683**	**0.1212**	-0.0116	-0.0146	-0.0014	-0.0427	0	0.7258
Luling	**0.1469**	**0.0822**	**0.1551**	0.0109	0.0154	0.0135	0.0081	-0.0116	0

Table 4.12. Comparison between observed R_{ST} values and the mean expected values of R_{ST} (pR_{ST}) after allele size randomization; CI, confidence interval.

	R_{ST}	pR_{ST} (95% CI)	p
All loci	0.0709	0.0081 (-0.0034-0.0257)	<0.0000
QfA130	0.0156	0.0145 (-0.0010-0.0597)	0.3952
QfC109	0.0039	0.0081 (-0.0102-0.0315)	0.6017
QfD103	0.0055	0.0108 (-0.0098-0.0432)	0.5808
QfC6	0.1200	0.0059 (-0.0114-0.0391)	<0.0000
QfC114	0.0099	0.0060 (-0.0092-0.0288)	0.2746
QfD5	0.0645	0.0072 (-0.0125-0.0472)	0.0063

4.4.3 Assignment Test

The STRUCTURE analysis was performed for 1 to 9 populations with a burn-in period of 10^4 repetitions, 5×10^4 repetitions for the MCMC run, and runs were repeated 20 times. Using the default setting (i.e., without LOCPRIOR) the level of genetic differentiation between the Guadalupe/San Marcos and the San Antonio rivers was not strong enough to distinguish separate populations - estimated values for LnP(D) were maximized at K = 1. Using the LOCPRIOR setting, which uses sample location as a prior for assisting the clustering, produced estimated values for LnP(D) that were maximized at K = 2 (Figure 4.7a). The ΔK statistic also shows a strong inflection point at K = 2 (Figure 4.3b) and lends support to an estimate of 2 population clusters. Moreover, at K = 2 the defined clusters correspond to the San Antonio and Guadalupe/San Marcos drainage basins (Figure 4.8) which supports the previous results from the AMOVA analysis and pairwise difference estimates.

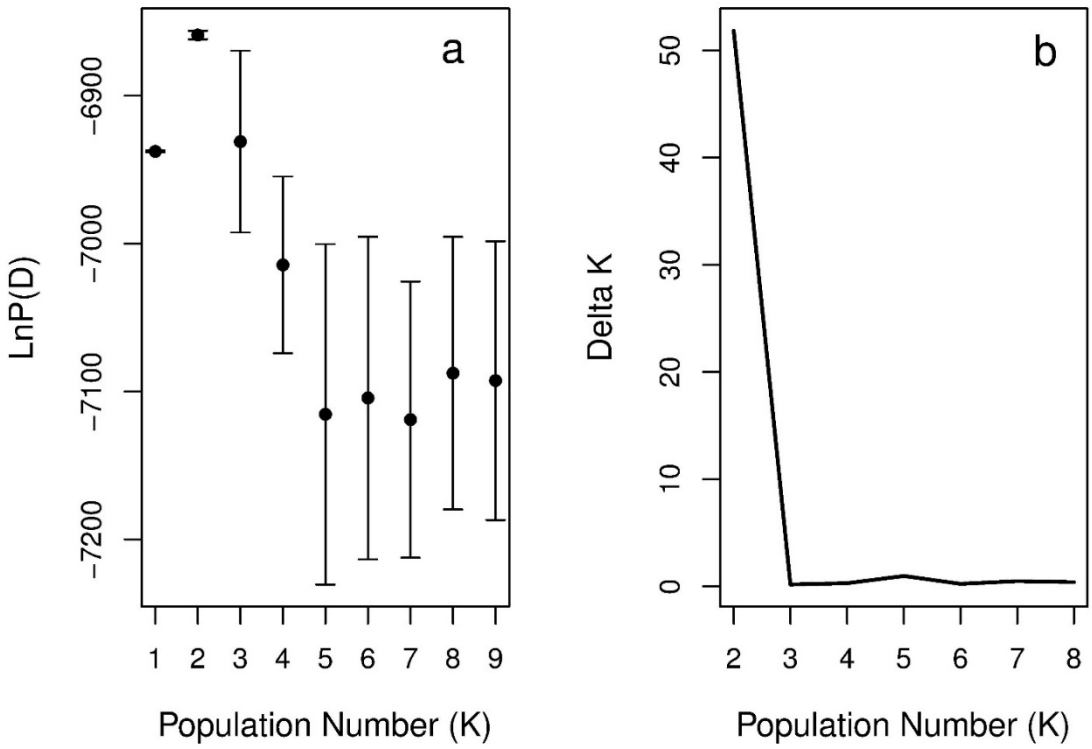

Figure 4.7. Results of the STRUCTURE analysis: (a) log probability of the data as a function of K and (b) magnitude of the ad-hoc statistic Δ K as a function of K.

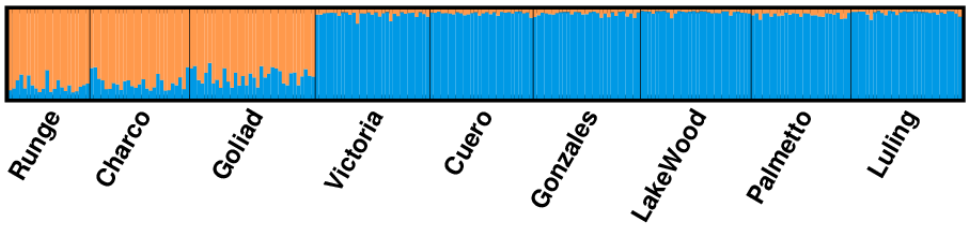

Figure 4.8. Bar plot obtained from the STRUCTURE analysis, using the LOCPRIOR setting, detailing the estimated membership coefficients ($\hat{Q}$) for individuals in 2 groups (K = 2): Group 1 (blue) includes populations from the Guadalupe and San Marcos Rivers and Group 2 (orange) includes populations from the San Antonio River.

4.5 Population Size

The results of the genetic structure analyses indicated populations within drainage basins are panmictic. Therefore, we grouped populations by drainage basin for the bottleneck assessment and the MSVAR analysis. Applying an α-level of 0.05, the bottleneck analysis was

not significant for either population regardless of mutation model (Table 4.13) and suggests there is no clear genetic evidence for a recent reduction in N_e.

Table 4.13. P-values for the Wilcoxon Sign-Rank Test for the BOTTLENECK analysis. IAM, Infinite Alleles Model; SMM, Stepwise Mutation Model; TPM, Two Phase Model.

	IAM	SMM	TPM
San Antonio	0.2188	0.9766	0.2188
Guadalupe/San Marcos	0.0781	0.9922	0.2813

The MSVAR analyses suggest the current effective population size (N_0) of *Q. aurea* has changed from the ancestral condition (N_1) in both the Guadalupe/San Marcos and San Antonio rivers (Figure 4.9). However, the direction of change was not consistent between river basins; results for the San Antonio River indicate a population reduction relative to historic levels while results for the Guadalupe/San Marcos indicate an increase in population size. Posterior distributions for both N_0 and N_1 for the Guadalupe/San Marcos were relatively consistent across runs despite starting values for N_1 that varied by 2 orders of magnitude. Posterior distributions for N_0 for the San Antonio River varied more than estimates of N_1 and several runs produced a left hand tail that implies a greater degree of uncertainty in the estimates of current effective population size (N_0) for the San Antonio River. The Gelman Rubin statistic was less than 1.1 in all analyses, which indicates convergence was obtained (Table 4.14). However, autocorrelation values were high for N_0 and X_a in the San Antonio analyses and X_a in the Guadalupe analyses, which suggests mixing was poor in these estimated parameters.

Median estimates for the current effective population size (N_0) in the Guadalupe/San Marcos drainage averaged approximately 78,000 and point to a large population (Table 4.15).

The current effective population size (N_0) is 3.5 times greater than the average estimated historic size (N_1) of approximately 22,000. Estimates for the mean effect sizes (g) for the change in population size ranged from 1.75 to 1.97 standard deviations (Table 4.14) which could be considered a large effect (Cohen 1988). Moreover, none of the 95% confidence intervals for *d* included zero, which indicates all the analyses were statistically significant.

Table 4.14. Diagnostic statistics for the MSVAR analysis. Gelman, Gelman-Rubin statistic; g, effects size; AC, autocorrelation function at 75th quartile (67,500 samples).

	Gelman	g	g Upper 95%	g Lower 95%	N_0 AC	N_1 AC	X_a AC
Guad/San Marcos							
Prior set 1	1.02	1.197	1.207	1.187	0.58	0.35	0.72
Prior set 2	1.04	1.175	1.185	1.165	0.57	0.43	0.73
Prior set 3	1.04	1.179	1.189	1.169	0.53	0.54	0.73
San Antonio							
Prior set 1	1.01	-1.295	-1.285	-1.305	0.71	0.08	0.7
Prior set 2	1.05	-1.203	-1.194	-1.212	0.73	0.15	0.71
Prior set 3	1.06	-0.850	-0.841	-0.860	0.72	0.3	0.7

Current effective population size (N_0) estimates for the San Antonio averaged about 5,500 and indicate a population reduction of approximately 86% from historic levels (Table 4.15). The effects size analysis again suggested a large and statistically significant change (approximately 1.12 standard deviations). However, it is important to note the uncertainty in the posterior distribution of N_0 for the San Antonio River, which was heavily skewed to the left in several model runs (Figure 4.9). A few runs produced tighter posterior distributions for N_0 (Figure 4.9 e and f) with modes suggesting a N_0 of approximately 4.2 or 16,000.

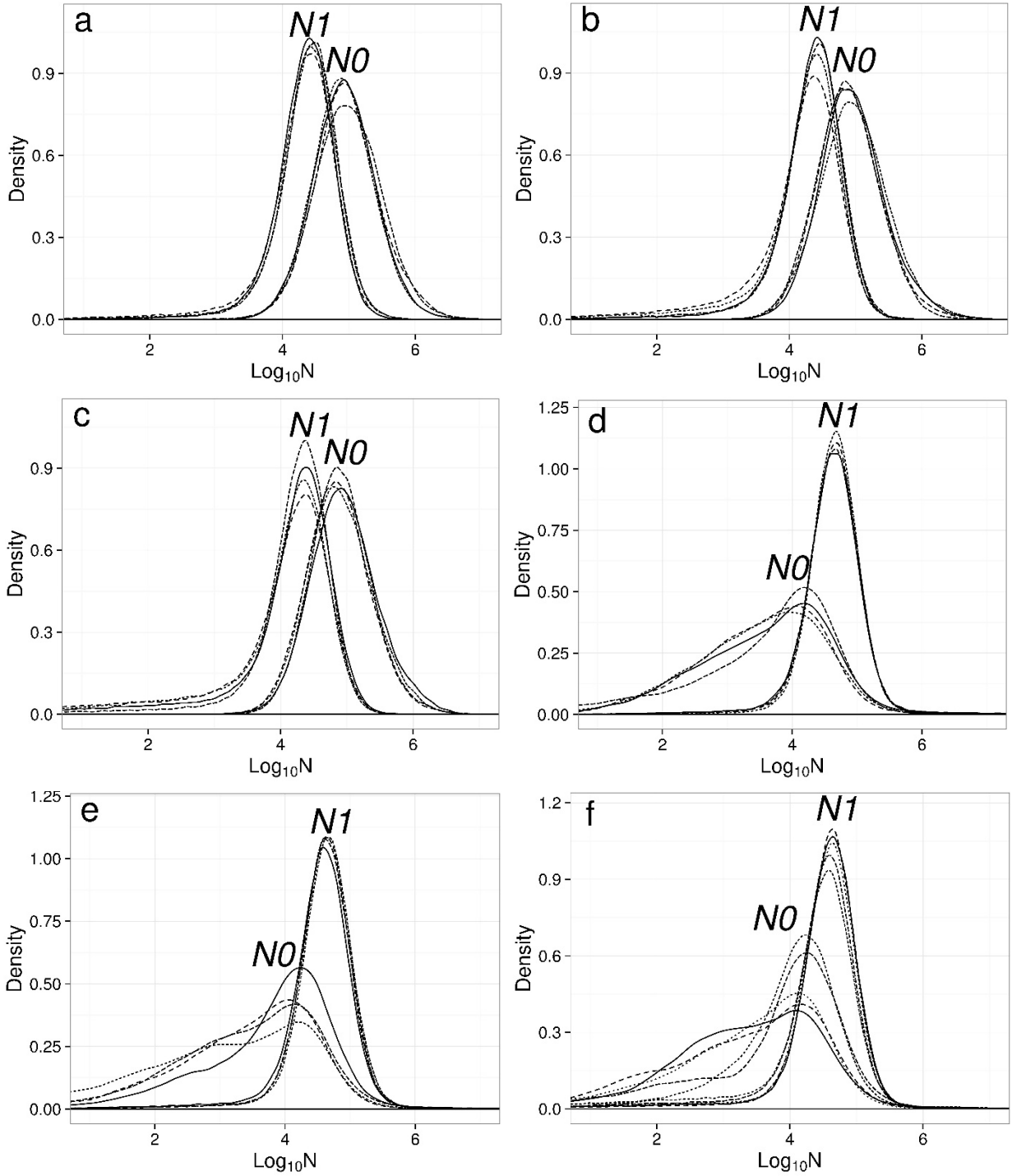

Figure 4.9. Posterior distributions for N_0 (current population size) and N_1 (ancestral population size) from the MSVAR analyses. Runs for the Guadalupe/San Marcos are identified as a, b, and c (prior sets 1, 2, and 3 respectively) while runs for the San Antonio are identified as e, d, and f (prior sets 1, 2, and 3 respectively).

Posterior distributions for the time since population change (X_a) were similar to the

effective population size distributions in that they were relatively consistent for the Guadalupe/San Marcos and more variable for the San Antonio (Figure 4.10). The median estimated time since the change in effective population size also varied between the Guadalupe/San Marcos and San Antonio rivers (Table 4.15). While the expansion in the Guadalupe/San Marcos appears to relatively ancient, having occurred during the Pleistocene around 40,000 years ago, the reduction in the San Antonio may have occurred as recently as 1,800 years ago. A high degree of variation in the posterior distribution for X_a again provides more uncertainty in the estimate for the time since change in the San Antonio River. The same runs that produced higher estimates for N_0 in the San Antonio also suggest the time since change was more ancient than the overall analysis suggests.

Table 4.15. Median, 10, and 90% quantiles for posterior distributions of the MSVAR parameters N_0, current population size; N_1, ancestral population size; X_a, time since population change.

	N_0 50% (10 – 90%)	N_1 50% (10 – 90%)	X_a 50% (10 – 90%)
Guad/San Marcos			
Prior set 1	85,114 (19,054 – 371,535)	25,119 (5,888 – 77,625)	38,019 (8,710 – 229,087)
Prior set 2	75,858 (16,982 – 363,078)	22,387 (3,388 – 70,795)	38,905 (7,943 – 371,535)
Prior set 3	74,131 (18,621 – 338,844)	18,621 (794 – 63,096)	44,668 (9,550 – 794,328)
Mean	78,368	22,042	40,531
San Antonio			
Prior set 1	5,623 (145 – 51,286)	43,652 (13,183 – 131,826)	1,862 (30 – 199,526)
Prior set 2	4,677 (69 – 45,709)	40,738 (11,749 – 120,226)	1,349 (15 – 154,882)
Prior set 3	6,310 (95 – 51,286)	36,308 (6,607 – 109,648)	2,291 (18 – 1,288,250)
Mean	5,537	40,232	1,834

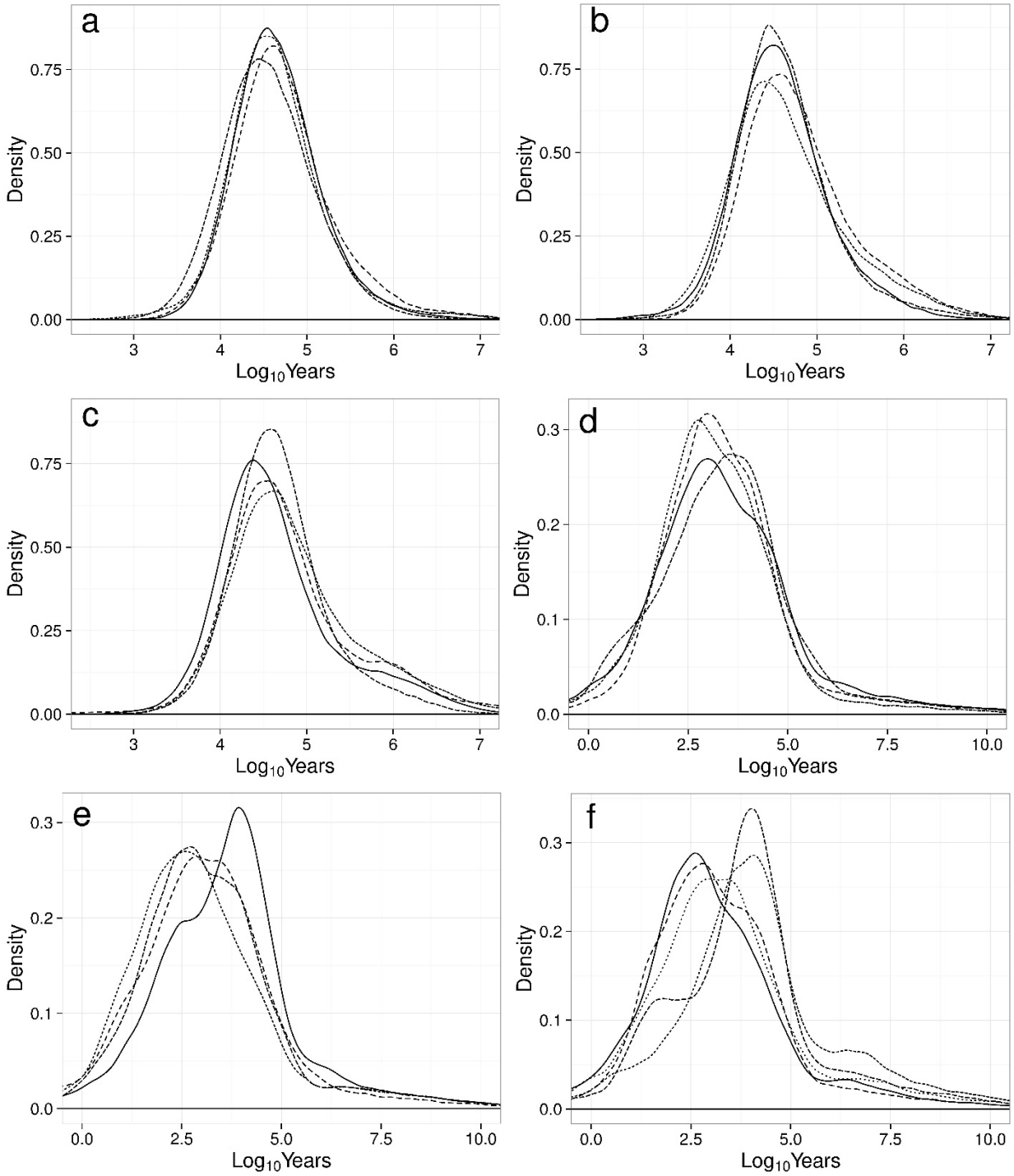

Figure 4.10. Posterior distributions for X_a (time since population size change) from the MSVAR analyses. Runs for the Guadalupe/San Marcos are identified as a, b, and c (prior sets 1, 2, and 3 respectively) while runs for the San Antonio are identified as e, d, and f (prior sets 1, 2, and 3 respectively).

4.6 Influence of Spatial and Environmental Factors on Genetic Structure

Q. aurea populations show a significant correlation between geographic distance and genetic distance in (r = 0.80, p = > 0.0029; Figure 4.11). The overall correlation appears to reflect the level of differentiation between the San Antonio and Guadalupe/San Marcos drainages. When the pairwise comparison was partitioned into within (populations within the same drainage; Figure 4.12) and between (populations in different drainages; Figure 4.12) categories, no significant correlations were evident (r = 0.09, p = 0.3585 and r = 0.37, p = .0654 respectively). The lack of a significant correlation in the between category was largely driven by the middle site in the San Antonio drainage, Charco, which consistently showed more genetic similarity with the Guadalupe/San Marcos sites than the other San Antonio River sites.

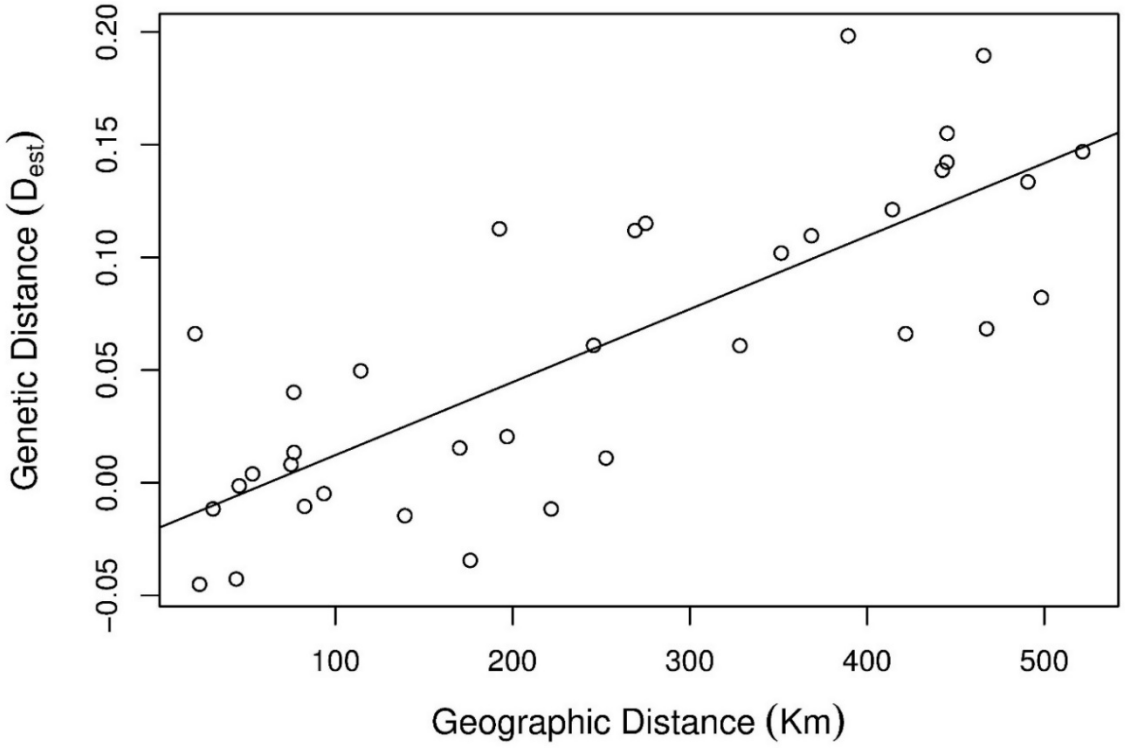

Figure 4.10. Correlation of geographic (river) distance verses genetic (D_{est}) distance for the freshwater mussel *Quadrula aurea* (*r* = 0.80, *p* = 0.0029). *P*-values calculated using a Mantel test.

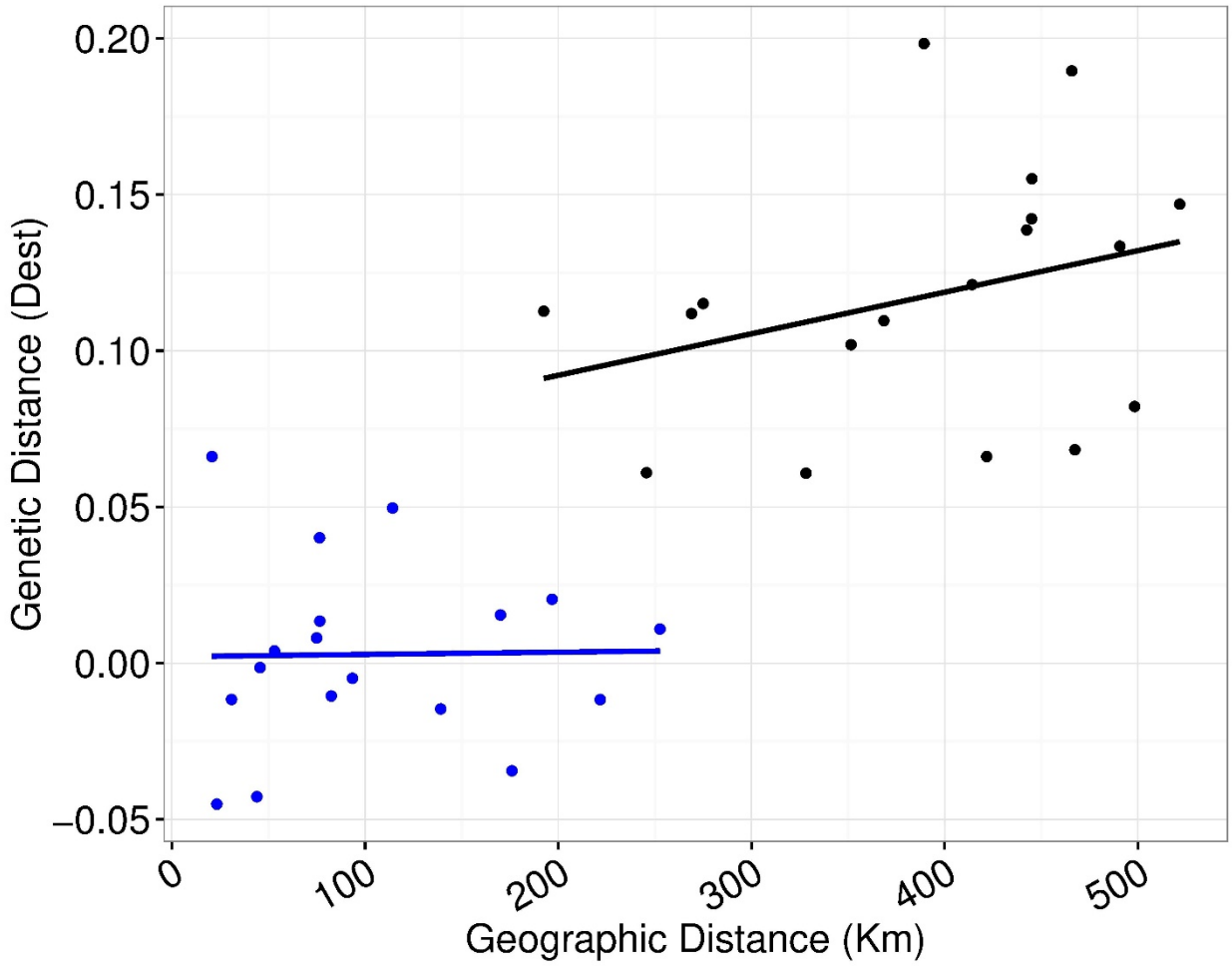

Figure 4.11. Correlation of geographic (river) distance verses genetic (D_{est}) distance for the freshwater mussel *Quadrula aurea* by category; (a) among populations within the same drainage (r = 0.09, p = 0.3585); (b) among populations in different drainages (r = 0.37, p = .0654). *P*-values calculated using a Mantel test.

The results of the Sunder analysis comparing geographic distance to environmental distance models based solely on landuse or soil composition indicate geographic distance has a stronger influence on genetic population structure (Table 4.16). In all runs of the analysis the model utilizing only geographic distance had a higher likelihood than either the landuse model, the soil composition model, or the combined models (geographic distance and landuse or geographic distance and soil composition).

Table 4.16. Results of the Sunder analysis using environmental distances based solely on landuse or soil composition within 10 km of each sampling site. Models with the highest likelihood for each run are in bold type. G, geographic; E, Environmental, G+E, combined geographic and environmental; NA, not applicable.

Model	Alpha	Beta_G	Beta_E	Gamma	Delta	Likelihood	Run
Landuse							
G+E	0.891	1448.6	19.91	0.947	0.00393	-740.2471	1
G+E	0.945	1345	21	0.967	0.0041	-614.9769	2
G+E	0.881	1578.9	18.85	0.971	0.004	-581.5194	3
G	**0.799**	**1637.4**	**NA**	**0.961**	**0.00419**	**-732.2665**	**1**
G	**0.82**	**1381.6**	**NA**	**0.963**	**0.00415**	**-599.7645**	**2**
G	**0.759**	**1779.9**	**NA**	**0.964**	**0.00431**	**-572.1118**	**3**
E	0.88	NA	18.28	0.964	0.00453	-743.2186	1
E	0.817	NA	19.7	0.948	0.00479	-607.7049	2
E	0.848	NA	16.81	0.965	0.00448	-588.1085	3
Soil Composition							
G+E	0.878	1345.9	403.6	0.957	0.00402	-617.9251	1
G	**0.793**	**1427.7**	**NA**	**0.965**	**0.00429**	**-615.5173**	**1**
E	0.868	NA	358.4	0.973	0.00388	-618.863	1

The results using the environmental distance model developed from the PCA analysis were mixed (Table 4.17) – the model utilizing only geographic distance produced the highest likelihood in 4 out of 10 runs, the model using only environmental distance was highest in 3 out of 10 runs, and the mixed model was highest in three out of 10 runs. However, a more thorough analysis of the relations between the distance matrices suggests *Q. aurea* population genetic structure is primarily influenced by geographic distance and drainage basin affiliation. Figure 4.13 depicts the rate of decay of genetic covariance as a function of geographic distance. Covariance clearly starts to decay as a function of distance, but it also appears to decay as a function of drainage affiliation; there is little decay among sites within the same drainage, but

covariance decays significantly among sampling sites in different drainages. The genetic covariance function also decays as a function of environmental distance (Figure 4.14), but the pattern of decay is similar to the geographic distance model (i.e., little decay among sites in the same drainage and significant decay among sites in different drainages. Moreover, a correlation analysis comparing geographic distance and environmental distance based on the PCA habitat variables (Figure 4.15) indicates a relatively high correlation (r = 0.67) and suggests the relevant variation in environmental variables is largely a function of differences between river basins.

Table 4.17. Results of the Sunder analysis using environmental distance based solely on the habitat variables chosen as a result of the principle components analysis. Models with the highest likelihood for each run are in bold type. G, geographic; E, Environmental, G+E, combined geographic and environmental; NA, not applicable.

Model	Alpha	Beta_G	Beta_E	Gamma	Delta	Likelihood	
G+E	0.85	1915.2	26.9	0.945	0.00363	-674.4048	1
G+E	0.873	1691.3	25.1	0.966	0.00397	-564.0071	2
G+E	**0.839**	**1738.3**	**22.9**	**0.966**	**0.00375**	**-538.0088**	**3**
G+E	0.922	1339.8	24.3	0.969	0.00361	-636.3667	4
G+E	0.94	1612.1	28.5	0.961	0.00398	-762.7156	5
G+E	0.891	1585.6	21.8	0.969	0.00376	-615.0589	6
G+E	0.933	1423.2	22.8	0.968	0.00395	-657.5539	7
G+E	**0.904**	**1675.2**	**23.7**	**0.971**	**0.00381**	**-686.9955**	**8**
G+E	0.928	1386.5	16.2	0.714	0.00406	-590.6752	9
G+E	**0.889**	**2175.2**	**26.6**	**0.972**	**0.00369**	**-687.9547**	**10**
G	0.762	1254.9	NA	0.966	0.00388	-676.154	1
G	**0.814**	**1462.6**	**NA**	**0.959**	**0.00479**	**-559.1342**	**2**
G	0.785	1719.2	NA	0.959	0.00439	-541.4897	3
G	0.799	1362.6	NA	0.955	0.00403	-630.8601	4
G	**0.846**	**1480.8**	**NA**	**0.961**	**0.00429**	**-754.1811**	**5**
G	**0.828**	**1614.1**	**NA**	**0.96**	**0.00406**	**-601.07**	**6**

(table continues)

Model	Alpha	Beta_G	Beta_E	Gamma	Delta	Likelihood	
G	0.749	1697.6	NA	0.967	0.00408	-648.3961	7
G	0.8	1436.3	NA	0.96	0.00379	-690.4123	8
G	**0.76**	**1800.6**	**NA**	**0.963**	**0.00338**	**-570.4381**	**9**
G	0.772	1560.7	NA	0.947	0.00392	-692.7717	10
E	**0.769**	**NA**	**26.9**	**0.967**	**0.00399**	**-672.4989**	**1**
E	0.843	NA	20.1	0.974	0.00374	-563.8422	2
E	0.827	NA	25.8	0.976	0.00368	-541.8577	3
E	**0.783**	**NA**	**19.9**	**0.971**	**0.00399**	**-622.8117**	**4**
E	0.88	NA	24.7	0.966	0.00376	-762.1359	5
E	0.815	NA	26.6	0.956	0.00413	-602.4352	6
E	**0.859**	**NA**	**20.3**	**0.974**	**0.00389**	**-647.4563**	**7**
E	0.824	NA	20.1	0.975	0.00374	-697.7958	8
E	0.826	NA	20.9	0.975	0.00371	-576.9096	9
E	0.838	NA	22.2	0.969	0.00394	-691.2167	10

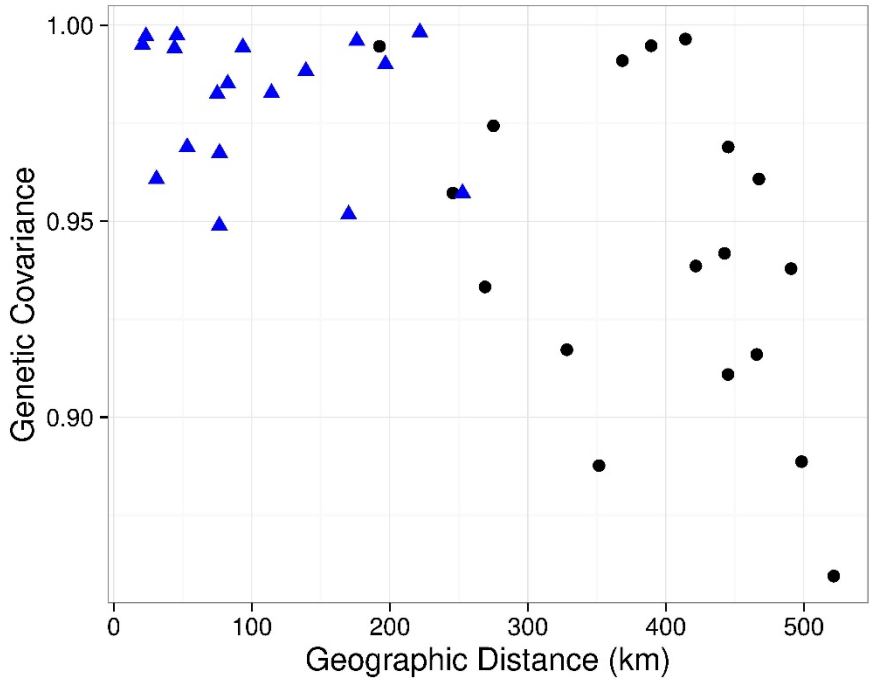

Figure 4.12. Genetic covariance as a function of geographic distance. Blue triangles represent distance values among sites within the same drainage (Guadalupe/San Marcos or San Antonio) while black dots represent distance values among site in different drainages.

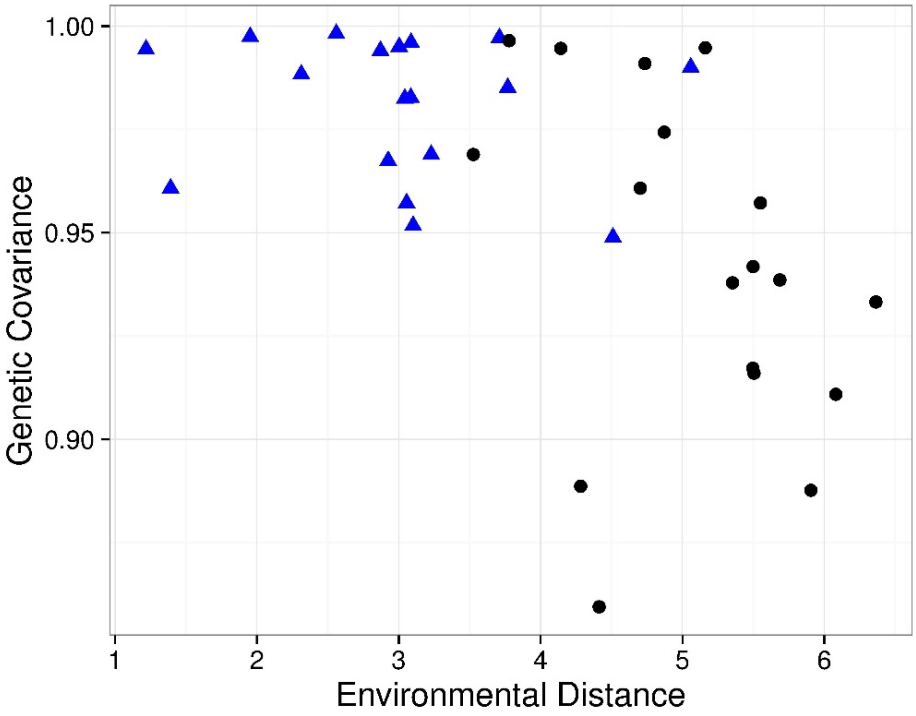

Figure 4.13. Genetic covariance as a function of environmental distance. Blue triangles represent distance values among sites within the same drainage (Guadalupe/San Marcos or San Antonio) while black dots represent distance values among site in different drainages.

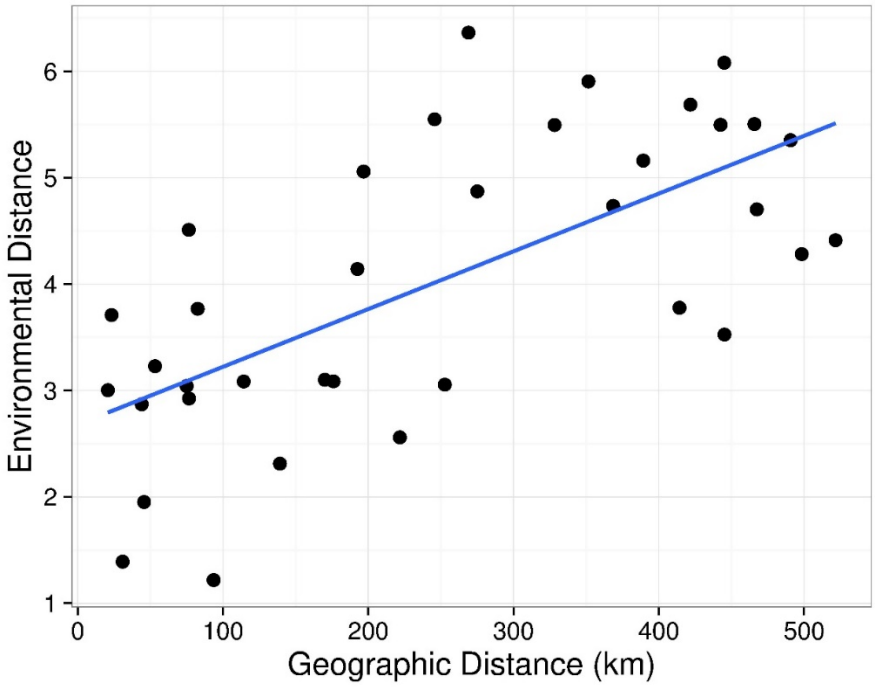

Figure 4.14. Correlation between geographic distance and environmental distance based on habitat variables selected with the PCR analysis.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Survey Results

Surveys conducted for this research were primarily focused on the collection of genetic material for the analysis of *Q. aurea* population structure. A secondary goal was to document the occurrence of other threatened or endangered unionids. Survey results generally support the perception of a decline in the occurrence of many endemic freshwater mussels in central Texas, but also provide some new insight into the distribution of remaining populations and abundance levels. The historic distribution of *Q. petrina* included the Colorado, Guadalupe, and San Antonio drainages (Howells 2010). As recently as 2010, this species was thought to persist in only two locations (Howells 2010). Surveys conducted for this project found *Q. petrina* in 9 separate locations (5 in the Guadalupe and 4 in the San Marcos; Appendix B) and some populations appear to be moderately abundant – 51 individuals were discovered at a remote site above Luling, TX in 1.5 person hours of searching. Based on the encounter rates found in this project (Table 4.1) the San Marcos, in particular, may harbor a significant number of the remaining *Q. petrina* individuals and should be an important factor in any strategy to conserve this species. On the other hand, surveys found no evidence of the continued existence of *Q. petrina* in the San Antonio drainage.

The false spike (*F. mitchelli*) is an endemic central Texas unionid bivalve that is currently under review for listing under the Endangered Species Act and was presumed extinct (Howells et al., 1996; Haag, 2009a; Howells, 2010) until a very-recently deceased individual was found on the San Saba River in 2011 (Randklev et al., 2011). Subsequent surveys produced seven live

specimens of *F. mitchelli* in one location on the Guadalupe River in south-central Texas (Randklev et al., 2011). These findings demonstrated the continued existence of *F. mitchelli* in central Texas, but it is important to note that all of the live specimens found were adults in the same size class. Freshwater mussels can be long-lived with life spans ranging from <10 to approximately 130 years (Haag, 2009b) and functionally extinct populations, composed solely of non-reproducing adults, have been reported from heavily altered river systems (Layzer et al., 1993; Hughes and Parmalee, 1999). The lack of a range of size classes in the only known extant population of *F. mitchelli* implied the reproductive status of the species was still questionable.

The discovery of a range of size classes in both populations of *F. mitchelli* found in this study, including some clearly juvenile specimens, indicate these populations have successfully reproduced recently. While the discovery of two reproducing populations of *F. mitchelli* is encouraging news for efforts to conserve the species, survey results also found no evidence for the continued existence of the species in the San Antonio or San Marcos Rivers (Table 4.1).

Identifying a reproducing population of *F. mitchelli* is of critical importance because very little is known about the species' biology or habitat requirements (Howells et al., 1996; Howells, 2010) and information is needed to facilitate the U.S. Fish and Wildlife review process for listing (USFWS, 2009). A reproducing population implies the presence of ecological conditions sufficient for population persistence and represents a rare opportunity to further our knowledge of the habitat requirements of this species. To this end, we measured a set of physical variables commonly assessed in riverine studies (i.e., wetted channel width, channel slope, flow velocity, water depth, and substrate composition) to document the general habitat conditions where the Cuero population was found. The results of that effort were published in

the Southwestern Naturalist (Vol 59, Issue 2, Pages 297-300) and are included as Appendix E in this document.

The historic range of *Q. aurea* likely included all of the Guadalupe, San Marcos, San Antonio, and Nueces-Frio river drainages (Howells 2010). Within its range the species was thought to be relatively rare (Howells 2010, Burlakova et al. 2011) and surveys conducted in the last few decades have documented the decline of the species in the upper reaches of the Guadalupe River (Howells 2010). In recent years, however, numerous populations have been discovered in the lower reaches of the Guadalupe (Burlakova and Karatayev 2010) and San Antonio rivers (Larralde 2011) including some of the largest known (Howells 1997, Burlakova and Karatayev 2010). Surveys conducted for this project suggest *Q. aurea* may be more abundant in the lower reaches of its historic range than generally thought. During this research 6 previously unknown populations were discovered and several had encounter rates (Appendix B) that indicated moderately large populations. More to the point, *Q. aurea* populations were generally encountered rather quickly during survey efforts. Considering only the six previously unknown populations, the mean distance traveled from survey starting point until encountering a population of sufficient size to sample for genetic material (at least 25 individuals >40 mm) was only 2.57 river kilometers. The fact that survey efforts for any particular reach were halted after encountering a population of sufficient size for genetic sampling suggests other populations were left undiscovered. Results of this research effort suggest the relatively remote reaches of the Guadalupe River that lie between Gonzales, TX and Victoria, TX may harbor significant populations of central Texas endemic unionids, including the highly endangered *F. mitchelli*, and should be considered important to any strategy for unionid conservation in Texas.

5.2 General Habitat Results

Targeted freshwater mussel species were found over a relatively wide range of habitat types, but *Q. aurea* beds with a high enough density for genetic sampling tended to be found in two habitat types – in mixed sand, gravel, and cobble substrates under moderate flow velocities or in fine sediments under low flow velocities. These general habitat types are consistent with the hypothesis that the interaction between hydrologic forces and substrate characteristics limit where mussel beds can persist over time (Hardison and Layzer, 2001; Gangloff and Feminella, 2007; Zigler et al., 2008; Allen and Vaughn, 2010). This interaction is captured in the complex hydrologic variable RSS which was well below the threshold for substrate movement (RSS ≥ 1) at all sites. This result is not surprising, however, given that measurements were taken under low flow conditions. Most work on complex hydrologic variables and freshwater mussel habitat suitability suggest conditions at high flow are critical for understanding habitat suitability (Allen and Vaughn 2010). The fact that mussel beds were present where we sampled leads to the assumption that RSS values under high flows at these locations will not be substantially above the threshold for substrate movement. Further study of these location during high flow conditions would verify or refute this assumption.

The purpose of the habitat data collected for this study was to compare population genetic structure to environmental setting and not to evaluate the habitat characteristics that determine mussel presence or abundance *per se*. Nevertheless, the habitat analysis does provide some insight into the environmental factors influencing *Q. aurea* presence and reach-wide abundance. Mussel bed width (BW) and the various sediment composition variables were the only variables found to be significantly different between the Guadalupe/San Marcos and

San Antonio rivers. While these variables were not significantly associated with encounter rates (data not shown) they are consistent with observations made during mussel sampling efforts. The lower reaches of the San Antonio River are dominated by loose sandy substrates (Engel 2007) which likely provide poor mussel habitat under high flow conditions (Gangloff and Feminella 2007, Allen and Vaughn 2010). Survey efforts during this study noted mussel presence in the San Antonio River was generally associated with the occurrence of rocky outcrops that provided larger particles to stabilize the sandy substrate. The width of mussel beds sampled in the San Antonio River were principally defined by the extent to which larger particles extended into the stream channel. In contrast the Guadalupe and San Marcos rivers are dominated by mixed sand, gravel, and cobble substrates (Phillips 2011, Strom et al. 2015) that provide for mussel beds that span the width of the river. The association of freshwater mussels with large particles that stabilize the otherwise mobile substrate has been documented by other researchers. Vannote and Minshall (1982) concluded that beds of *Margaritifera falcate* in the Salmon River were restricted to relatively stable cobble runs, but beds attained a maximum density where substrates were stabilized by large blocky boulders from the canyon walls. These researchers concluded that mussel beds associated with boulder fields were protected from periodic scour events that caused high mortality in unprotected beds. This hypothesis also fits with the general view that freshwater mussel occurrence tends to coincide with areas where sediments are stable during extreme flow events (Strayer 1999, Allen and Vaughn 2010).

5.3 Microsatellite Marker Identification

Microsatellite loci, because of their relatively rapid mutation rates, represent a powerful tool for characterizing population genetic structure, but marker sets used to amplify specific loci can be time consuming and costly to develop. Cross-species amplification of previously developed microsatellites can be a useful approach for reducing the cost and time required to identify microsatellite markers for many closely related species. However, cross-species transferability of microsatellites tends to decrease with increasing genetic distance between target species and source species (Wright et al. 2004, Barbará et al. 2007) and the utility of this strategy can be affected by various forms of ascertainment bias where amplification success and allelic diversity are reduced in non-source species (Wright et al. 2004, Li and Kimmel 2013). Loci that are polymorphic in the source species may be unresolved or monomorphic in target species (Eckles and King 2002, Nazia and Azizah 2014), amplification failure due to a mismatch in primer regions (i.e., null alleles) may be more common with evolutionary distance between species (Dakin and Avise 2004; Hedgecock et al. 2004), primers may amplify non-orthologous products (Yue et al. 2010), and size homoplasy (similarity in size between alleles that do not share an evolutionary history) can occur (Angers and Bernatchez 1997; Estoup et al. 2002).

Of the loci that have been reported to produce unambiguous polymorphic PCR products in *Q. fragosa*, over half were either monomorphic or unresolved in *Q. aurea*. A failure rate (either non-amplification or monomorphic) of approximately 50% is on the high end of cross-species amplification results reported for freshwater mussels (Eackles and King, 2002; Kelly and Rhymer 2005; Ward et al. 2010). The success rate of cross-species amplification is directly related to the evolutionary distance between species (Wright et al. 2004; Primmer et al. 2005;

Gupta et al. 2013). Thus, the lack of unambiguous PCR product could be the result of phylogenetic distance and accompanying divergence in primer regions between *Q. aurea* and *Q. fragosa*. Although *Q. aurea* and *Q. fragosa* belong to the same genus, taxonomists have used features of the shell (Simpson 1900) to place these species into separate species groups (Pustulosa and Quadrula respectively). Serb et al. (2003) demonstrated molecular support for this species group division within the genus *Quadrula* using a 700 base pair region of the first unit of the mitochondrial NADH dehydrogenase (ND1) gene, but were unable to include a specimen of *Q. fragosa* in their analysis. A direct comparison between *Q. aurea* and *Q. fragosa* would help to clarify the evolutionary distance between these species and provide insight into the potential mechanisms limiting the transferability of microsatellite between them. Interestingly, *Q. pustulosa* appears to be closely related to *Q. aurea* (Serb et al. 2003), but none of the four primers developed for *Q. pustulosa* produced unambiguous PCR products in *Q. aurea*.

5.4 Power Analysis

The power analysis indicates the overall study approach was sufficient to identify a true level of genetic divergence among *Q. aurea* populations of at least F_{ST} = 0.01. This value is essentially the level of genetic divergence that defines the genetic subdivision between the Guadalupe/San Marcos drainage and the San Antonio drainage (Table 4.7).

The uncertainty in genetic difference measurements such as F_{ST} tends to decrease with an increase in the number of microsatellite loci used (Kalinowski 2002). Consequently, modern studies focused on population structure commonly employ 10 to 20 or more loci (Kelson et al.

2015; Richmond et al. 2015). Microsatellite primer testing for this research effort only identified six microsatellites that functioned adequately in *Q. aurea* and it was feared that these results could limit the ability to identify significant population differentiation. However, the utility of individual microsatellite markers depends greatly on the level of polymorphism they display and a few high polymorphic markers can be as powerful as many less variable markers (Kalinowski 2002). The high degree of polymorphism displayed buy the microsatellites identified in this study coupled with the results of the power analysis indicate the six loci are adequate for the purposes of identifying contemporary population structure in *Q. aurea*.

Given the power of the analysis the lack of statistical significance between the Charco and Cuero sites (F_{ST} = 0.0095) seems anomalous. Especially given the fact that the difference between Charco and Victoria is statistically significant at F_{ST} = 0.0079. However, the pairwise analysis between Charco and Cuero was statistically significant under the measure of differentiation that utilized the stepwise model (R_{ST}; Table 4.7) and the measure designed to scale linearly with differences among populations (D_{est}; Table 4.8). This difference may reflect the influence of two markers, QfC6 and QfD5. In the case of R_{ST}, these markers appear to adhere more closely to a stepwise mutation model (Table 4.9). In general, the utility of R_{ST} is reduced when the pattern of mutation diverges from the stepwise mutation model (Slatkin 1995; Balloux et al. 2000). Nevertheless, if the mutation process produces new alleles that are more similar in size to the previous state (in comparison to randomly chosen alleles) then R_{ST} may give a more accurate estimate of genetic differentiation than F_{ST} (Balloux and Lugon-Moulin 2002). In the case of D_{est}, the number of unique alleles associated with these hypervariable markers created relatively large locus-specific differences between Charco and

Cuero at the QfC6 and QfD5 loci. Because D_{est} is related to the proportion of alleles unique to each subpopulation – and not related to within-population diversity as F_{ST} is – allelic variation at hypervariable markers may carry more weight, so to speak, in the D_{est} analysis.

5.5 Genetic Structure

This study was initiated to evaluate the neutral genetic diversity and population structure of the threatened freshwater mussel *Quadrula aurea* in the lower reaches of the Guadalupe, San Marcos, and San Antonio rivers. Our results indicate *Q. aurea* populations contain substantial within-population neutral genetic diversity; mean values for both expected heterozygosity (H_E) and allelic richness (A_R) were high in all populations and only one loci (QfC109) showed relatively low levels of polymorphism. Studies focused on the genetic structure of threatened freshwater mussels have shown a range of neutral genetic diversity (Kelly and Rhymer 2005; Zanatta and Murphy 2008; Geist et al. 2010; Mock et al. 2010; Grobler et al. 2011; Inoue et al. 2014; Karlsson et al. 2014; Jones et al. 2015) and the results for *Q. aurea* are on the higher end of this range. However, direct comparisons between different microsatellite loci can be misleading due to variation in the characteristics (complexity, repeat motif, mutation rate, etc.) of loci with different evolutionary histories. Roe and Boyer (2015) also used four of the six loci used to evaluate *Q. aurea* (OfA130, QfC109, QfC6, and QfD5) to compare the genetic diversity of two other closely related species: the rare and endangered *Quadrula fragosa* and the widespread and abundant *Amphinaias pustulosa*. Considering only these four loci, mean allelic richness (A_R) across all populations of *Q. aurea* (12.3, n = 22) falls between the values reported by Roe and Boyer (2015) for *A. pustulosa* (15, n = 39) and *Q.*

fragosa (10.1, n = 39) despite a smaller rarefaction size for *Q. aurea*. Locus specific heterozygosity (H_E) values for *A. pustulosa* were not reported and could not be compared, but the range of mean H_E values for the four common microsatellites across all *Q. aurea* populations (0.76-0.83; Table 4.5) brackets the mean (0.80) for the single *Q. fragosa* population studied by Roe and Boyer (2015).

One of the primary objectives of this study was the assessment of genetic connectivity/isolation among *Q. aurea* populations both within and among drainage basins. Our results indicate a high level of connectivity among *Q. aurea* populations. Populations within the same drainage were not significantly different from one another and displayed a homogeneous panmictic structure with little genetic differentiation. Nevertheless, *Q. aurea* populations separated by the confluence of the San Antonio and Guadalupe/San Marcos drainages showed weak genetic differentiation. This pattern was evident in the AMOVA analyses and all three pairwise fixation indices (F_{ST}, R_{ST}, and D_{est}) despite a set of microsatellite markers with varying levels of conformity to specific mutation model assumptions (i.e. the IAM or the SMM). While we found an overall IBD pattern, its statistical significance was driven primarily by the pairwise comparisons among populations from different drainages (i.e. the San Antonio vs. the Guadalupe/San Marcos), while pairwise comparisons within drainages showed no pattern of IBD.

On the other hand, the assignment tests using the STRUCTURE program demonstrate the overall weakness of this structural pattern. The STRUCTURE runs using only the genetic data were unable to identify population differentiation. A maximum likelihood of two populations (k = 2), corresponding to the Guadalupe/San Marcos and the San Antonio, was only achieved in

STRUCTURE under the LOCPRIOR setting. The necessity of using the sampling locations as priors for the clustering algorithm suggests gene flow may still be relatively high between the Guadalupe/San Marcos and San Antonio.

The genetic differentiation documented among *Q. aurea* populations in this study is small in comparison to other studies of freshwater mussels that utilized microsatellites and a comparable study area (Kelly and Rhymer, 2005; Geist et al. 2010). For example Kelly and Rhymer (2005) documented significant within-basin F_{ST} values of up to 0.100 and found significant population structure at distances of only 36 kilometers. On the other hand, values reported by this study correspond to the low end values of other microsatellite studies that suggest high gene flow among freshwater mussel populations (Inoue et al. 2014; Jones et. al 2015).

The genetic structure of freshwater mussels may be affected by several factors including geographic history, dispersal ability, N_e, and life history traits. Common ancestry is implied by the small range size and endemic nature of *Q. aurea* so a relatively low level of genetic divergence among populations could be expected. However, common ancestry does not necessarily translate to panmixia in microsatellites. Zanetta and Murphy (2007) cited common ancestry, related to expansion from refugia after the last glacial maximum, as the reason for low mitochondrial genetic structure in *Epioblasma torulosa rangiana*, but found significant microsatellite structure on a scale of only 15 km. In contrast, Inoue et al. (2014) suggested two mitochondrial lineages found in *Cumberlandia monodonta* were the product of common ancestry, but low divergence across large spatial scales for both mitochondrial and microsatellite markers was likely the result of high gene flow.

While *Q. aurea* is likely affected by common ancestry, some evidence suggests the present genetic structure is more likely related to gene flow. The strong (r = 0.80) IBD pattern that exists across drainage basins suggests populations have been in place long enough to develop a regional equilibrium (Hutchison and Templeton, 1999). A pattern of IBD reflects the differential influence of migration and drift as the distance increases between populations. Migration maintains similar allele frequencies among populations in close proximity while allele frequencies among more distant populations that exchange less migrants will tend to drift apart. Isolation by distance should develop if populations have been established for a sufficient time without significant barriers to dispersal (Hutchison and Templeton, 1999). Rivers in the tectonically inactive Texas Gulf Coast region were far removed from the direct effects of Holocene glaciation (Blum and Aslan 2006; Galloway et al. 2011) and have likely provided sufficient long-term stability and connectivity for the development of regional equilibrium between the San Antonio and Guadalupe/San Marcos systems. The relatively even distribution of many unique, low frequency, alleles across all populations also suggests gene flow as a driving factor for genetic structure. Mutation and homoplasy can also account for the presence of some low frequency alleles in both drainages. On the other hand, the distribution of several low frequency alleles, which were found across the interior populations and missing from populations at the extremes of the study area, suggests gene flow moving out from an original source mutation.

Dispersal ability exerts a strong influence on genetic connectivity and transport by host fish in the glochidial phase is an important component of unionid dispersal. As a consequence dispersal distance and connectivity are often closely tied to host fish movement (Zanatta and

Wilson 2011; Schwalb et al. 2011; Karlsson et al. 2014). Recent identification of the channel catfish (*Ictalurus punctatus*) as a primary host for *Q. aurea* (per. comm., Nathan Johnson, USGS Southeast Ecological Science Center) means *Q. aurea* populations may be connected by a highly mobile host species. Channel catfish have been documented traveling distances of up to 100 km or more (Funk 1957; Welker 1967; Becker 1983; Gerhart and Hubert 1990; Pellett et al. 1998) and may move up and downstream with similar frequency (Funk 1957; Welker 1967; Dames et al. 1989, Wendel and Kelsch 1999). High connectivity among *Q. aurea* populations would work to maintain gene flow and genetic diversity, and homogenize allele frequencies across large spatial scales. Because relatively little gene flow is required to counteract genetic drift and prevent substantial population subdivision (Mills and Allendorf 1996; Freeland et al 2011), few migratory events involving glochidia infected channel catfish would be sufficient to maintain panmixia among widely dispersed *Q. aurea* populations.

Genetic differentiation between the San Antonio and Guadalupe/San Marcos drainages may reflect the natural movement patterns of channel catfish. Long distance movements of channel catfish occur seasonally in relation to spawning behavior (Pellet et al 1998; Butler and Wahl 2011) or high flow events (Cross 1950, Wendel and Kelsch 1999; Cathcart 2011) and are often cyclical in nature; fish move downstream in the autumn to overwinter in deeper water and upstream in the spring to spawn in shallower reaches. Some evidence points to channel catfish “homing” or returning to the same area each summer (Pellet et al. 1998, Butler and Wall 2011). A lack of movement between drainages due to cyclical or homing behavior could reduce gene flow between drainages and facilitate genetic differentiation. On the other hand, habitat conditions near the confluence of the San Antonio and Guadalupe/San Marcos drainages reflect

low gradient coastal conditions with fine, loose, substrate and sluggish flow. Our survey efforts found no *Q. aurea* populations near the confluence of the San Antonio and Guadalupe/San Marcos drainages. A lack of suitable habitat to support stepping stone populations near the confluence could also restrict gene flow between drainages.

The view that *Q. aurea* genetic structure is influenced by channel catfish vagility fits into an emerging picture of Unionoida genetic structure that suggests genetic divergence is strongly influenced by the life history characteristics of host fish. Highly vagile host fish have been cited as a potential explanation for limited genetic structuring across large geographic areas in other mussel species (Berg et al. 1998, Elderkin et al. 2007; Inoue et al 2014). In contrast, mussels that utilize relatively sedentary host species tend to exhibit significant genetic structure on relatively small spatial scales (Berg et al. 2007; Zanetta and Murphy 2007). Genetic structure of the critically endangered pearly mussel *Margaritifera margaritifera* in Norway differed significantly between populations that used different host fish (Karlsson et al. 2014). Populations that utilized anadromous Atlantic salmon (*Salmo salar*) had higher genetic diversity (H_E = 0.649, A_R = 4.667) and weaker structure (F_{ST} = 0.023) than regionally sympatric populations that utilized non-anadromous brown trout (*Salmo trutta*; H_E = 0.317, A_R = 2.252; F_{ST} = 0.332). Disconnects between host dispersal ability and mussel structure have been reported, but may reflect a lack of coordination between life history events and not host dispersal ability *per se*. For example, Kelly and Rhymer (2005) suggested the relatively high genetic differentiation they found among populations of *Lampsilis cariosa* did not reflect the dispersal potential of their probable host species, the white perch (*Morone americana*) and the yellow perch (*Perca flavescens*). These authors pointed out that the majority of dispersal in *P. flavescens* occurs during the larval stage,

which is not parasitized, while juveniles and adults that are capable of transporting glochidia maintain smaller home ranges (Miller 2003).

Another potential factor effecting genetic structure and diversity is a large effective population size (N_e). *Q. aurea* is generally thought to be rare to uncommon throughout its range (Howells et al. 1996, Burlakova et al. 2011) and surveys in recent years support the assumption of low numbers in the northwestern part of its range in the Edwards Plateau ecoregion (Howells 2006). However, other efforts suggest the species may be more abundant within our study area in the East Central Texas Plains and Western Gulf Coastal Plain ecoregions (Howells 1996, Burlakova and Karatayev 2010, Larralde 2011). Our own survey results include the discovery of five previously unknown populations and produced moderately large (5,537) to quite large (78,368) estimates of current effective population size for the San Antonio and Guadalupe/San Marcos drainages respectively. Estimates of N_e for other freshwater mussels vary broadly depending on the time period of estimation (contemporary vs long-term) and the mutation rate assumed. Nevertheless, other authors have found high neutral genetic diversity coupled with moderate to large historic N_e in other endangered mussels that display panmixia. For example, *Cumberlandia monodonta* was once widespread and locally common throughout the Mississippi River system, but is now documented in only 20 streams across 10 states (Butler 2002). A recent study of five populations of *C. monodonta* by Inoue et al. (2014) found low genetic divergence among populations, high H_E (0.74 to 0.85), and point estimates of long-term N_e, based on a generalized mutation rate of 5×10^{-4}, that ranged from 1410 to 5547. Jones et al. (2015) found global H_E values of 0.85 and 0.84 and calculated per generation N_e (N_e/generation

time) values of 1323 (± 88) and 8704 (± 445) for endangered *Epioblasma brevidens* and *E. capsaeformis* respectively in the Tennessee and Cumberland Rivers.

5.6 Asymmetric Gene Flow

Our results indicate little detectable influence of riverine structure or dams on neutral genetic diversity. The dendritic structure of riverine ecosystems can influence the distribution of genetic diversity (Campbell Grant et al. 2007; Horreo et al. 2011; Alp et al. 2012) and directional gradients may be apparent if gene flow is asymmetric due to the force of gravity or barriers to movement (Morrissey and Kerckhove 2009; Junker et. al 2012). Moreover, diversity at nodes (i.e. the confluence of two drainages) may be elevated due to the receipt of migrants from segments with different allele frequencies (Campbell Grant et al. 2007). We found a marginal, non-significant, increase in diversity in the downstream direction in the San Antonio River, but otherwise found no evidence of asymmetric gene flow. For example, genetic diversity in the uppermost sampling site on the San Marcos (Luling; H_E = 0.83, A_R = 11.15) was not significantly different from the furthest downstream site on the Guadalupe River (Victoria; H_E = 0.82, A_R = 11.25) despite being separated by 252 river kilometers and 2 major dams. The Gonzales site, which sits below the confluence of the Guadalupe and San Marcos rivers, did have the highest expected heterozygosity (H_E; 0.84) and the lowest inbreeding coefficient (F_{IS}; -0.058). These results are consistent with the theory of higher genetic diversity at the nodes of dendritic structures (Campbell Grant et al. 2007), but the results, again, were not statistically significant. The lack of a clear directional gradient in genetic diversity may reflect the historic ability of host fish (i.e., channel catfish) to move upstream prior to dam construction in the

early 1900's. On the other hand, the lack of a clear asymmetric signal could reflect a scale issue. The principle focus of this study was the quantification of genetic diversity and connectedness among *Q. aurea* populations in their region of highest abundance. Therefore collection efforts were focused on the mainstem sections of the lower San Antonio, Guadalupe and San Marcos Rivers. We recognize the likelihood of populations higher in these drainages; see Larralde 2011 for the San Antonio River and Howells 2006 for the Guadalupe River. It is possible that un-sampled populations higher in these drainages, if they are more isolated, may have significantly lower genetic diversity and represent clearly defined asymmetric gene flow in these systems.

5.7 Influence of Spatial and Environmental Factors on Genetic Structure

The Isolation by Distance (IBD) hypothesis, first described by Sewell Wright (1943) represents the idea that the level of gene flow between two populations is dependent on geographic distance – gene flow tends to be higher between populations that are close together and lower between populations that are farther apart. Under IBD local genetic differences accumulate as a result of reduced dispersal between populations and genetic drift. Analysis of the genetic data relative to spatial and environmental factors suggests IBD influences *Q. aurea* genetic structure to some degree. The mantel test for IBD was highly significant and the Sunder analysis demonstrates genetic covariance among sampling sites decays as a function of geographic distance. IBD has been demonstrated in other mussel species on both large (Berg et al. 1998, Elderkin et al. 2008) and relatively small spatial scales (Berg et al. 2007, Galbraith et al. 2015). However, another spatial pattern is evident in the overall data. Categorization of both the Mantel test and the Sunder analysis demonstrates the

IBD pattern is related in some degree to river basin affiliation (the Guadalupe/San Marcos or the San Antonio) and no pattern exists in the genetic variation among populations within the same river basin. Other examples of "Isolation by Drainage" have been reported for freshwater mussels. Galbraith et al. (2015) demonstrate IBD patterns for several species in southwestern Ontario, Canada, across various spatial scales, but genetic divergence in all cases was clearly associated with variation between rivers. Inoue et al. (2013), investigating the phylogenetic relationships and evolutionary divergence of two species, *Obovaria jacksoniana* and *Villosa arkansasensis*, found weak IBD on small spatial scales and strong IBD across drainage basin divides.

Another important aspect of the landscape that can influence gene flow is environmental heterogeneity. An increase in genetic diversity in relation to increasing environmental distance (Isolation by Environment, IBE) may occur if gene flow rates are higher between similar environments. The genetic factors driving IBE are similar to IBD (i.e., reduced dispersal and drift), but the underlying processes inhibiting gene flow occur irrespective of geographic distance (Sexton et al. 2014). Genetic covariance among *Q. aurea* sampling sites also decayed with environmental distance (and river basin affiliation) suggesting that environmental heterogeneity between river basins may contribute to *Q. aurea* population genetic structure.

Disentangling geographic and environmental influences on genetic structure can be difficult because IBD and IBE are often correlated (Sexton et al. 2014) as they are in this analysis (Figure 4.9). While the factor that creates IBD is relatively straight-forward, the factors that could create IBE in the Guadalupe/San Marcos and San Antonio system are not clear. In their

recent review, Wang and Bradburd (2014) list four processes that can potentially generate IBE: biased dispersal, natural selection against immigrants, sexual selection against migrants and reduced hybrid fitness. Freshwater mussels are broadcast spawners so sexual selection against migrants is unlikely, but some form of dispersal bias or selection pressure could be possible. Since dispersal in freshwater mussel is mediated by host fish movement, biased dispersal could arise if host fish species have a preference for specific environments. A degree of bias against moving between the Guadalupe/San Marcos and San Antonio rivers would also account for the influence of basin affiliation on genetic covariance. On the other hand, differences in sediment composition could create a selection bias. The specific environmental factors that may contribute to IBE in this system are not clear, but the only environmental factors that were significantly different between river basins are the soil composition characteristics. Moreover, although statistical tests associated with this study could not identify significant differences between mussel bed sediment characteristic, the lower San Antonio River is generally dominated by finer substrates than the Guadalupe/San Marcos (Engel 2007, Phillips 2011, Strom et al. 2015). Freshwater mussels have shown variation in shell morphology in relation to sediment composition in both lacustrine (Bailey and Green 1988) and riverine (Hornbach et al. 2010) environments, but changes appear to represent phenotypic plasticity (Inoue et al. 2013) as opposed to selection for genetically fixed characteristics.

5.8 Changes in Effective Population Size

The MSVAR analysis produced strikingly different results for current effective population size (N_0) in the San Antonio and Guadalupe/San Marcos Rivers. The results for the San Antonio

River should be considered with caution due to the apparent poor mixing in the model (Table 4.14) and the larger uncertainty evident in the posterior distribution estimates (Figure 4.19). Nevertheless, the median values for the MSVAR analysis suggest a large difference in effective population size exists between basins (Table 4.15). The large estimate for N_0 in the Guadalupe/San Marcos River is quite a bit larger than effective population size estimates reported for other threatened mussels species (see discussion in 5.3 Genetic Structure). This estimate is, however, in keeping with other evidence that suggests the *Q. aurea* population in the lower Guadalupe may be larger than originally thought. For example, Howells (1997) used twenty 0.25-m^2 quadrats and a single 1.0-m^2 quadrat to estimate a census population size of 188,000 for *Q. aurea* in a 2.8 kilometer reach of the Guadalupe River downstream of Lake Wood near Gonzales Texas. Burlakova and Karatayev (2010) and Hammontree et al. (2012) also found evidence of relatively high densities of *Q. aurea* at specific locations in the Guadalupe and San Marcos rivers. Simulation studies of the MSVAR program have demonstrated the potential to produce a spurious signal of demographic expansion with asymmetric gene flow (Paz-Vinas et al. 2013). This is likely not the case for the Guadalupe River as genetic data showed no indication of asymmetric gene flow (see section 5.4 Asymmetric Gene Flow).

The lower estimate of N_0 for the San Antonio also makes sense in light of other habitat information from this system. The lower reaches of the San Antonio River are dominated by loose sandy substrates (Engel 2007) which likely provide poor mussel habitat under high flow conditions (Gangloff and Feminella 2007, Allen and Vaughn 2010). Survey efforts during this study noted mussel presence was generally associated with the occurrence of rocky outcrops that provided larger particles to stabilize the sandy substrate. Given that these outcrops are

discontinuous throughout the lower San Antonio River, optimum habitat for mussels likely reflects a patchy distribution. Moreover, because the width of mussel beds in the San Antonio River were principally defined by the extent to which larger particles extended into the stream channel, overall mussel bed size is limited in the San Antonio River. Suitable mussel habitat in the San Antonio River appears to be limited in comparison to the Guadalupe/San Marcos River which is dominated by mixed sand, gravel, and cobble substrates (Phillips 2011, Strom et al. 2015) that provide abundant stable mussel habitat almost continuously down to the geomorphic transition into the Guadalupe delta (Phillips 2011). Larralde (2011) also found *Q. aurea* presence and abundance was highest in locations of mixed (gravel and sand) substrate, but made no mention of these locations being associated with rock outcroppings.

This study did not attempt to collect census data in a manner that would allow the estimation of the ratio of effective population size (N_e) to the census population size (N_c). Estimating this ratio for even a single species is complicated by uncertainty in the estimates of both parameters and the stability of N_e/N_c ratios over time (Luikart et al. 2010). Nevertheless, several authors have attempted to develop estimates for average (Frankham 1995) or median (Palstra and Ruzzante 2008; Palstra and Fraser 2012) N_e/N_c ratios across a wide variety of animal taxa. These efforts suggest "typical" N_e/N_c ratios could fall within a range from 0.10 to 0.23. Applying N_0 estimates for *Q. aurea* to this range of "typical" N_e/N_c ratios indicates the census population of *Q. aurea* in the lower Guadalupe/San Marcos and San Antonio Rivers could range from 339,130 to 780,000 and 23,913 to 55,000 respectively. While these numbers are based on extrapolation from other sources and *are not meant to be an accurate estimate of*

the census sizes for Q. aurea in these systems, they do provide some perspective on the potential size of populations if the MSVAR results represent an accurate estimate of N_0.

The MSVAR results for the Guadalupe/San Marcos basin suggest an increase in effective population size that started during the late Pleistocene approximately 40,000 year before the present time (ybp) (Table 4.15, Figure 4.10). Even lower end estimates (10% quantile) of timing of change average 8734 ybp. The results for the Guadalupe River differ from historic demographic changes identified in other freshwater mussel populations which have generally been associated with expansion out of refugia (Elderkin et al. 2007, Zanatta and Murphy 2008, Inoue 2014) or contraction due to isolation (Mock et al. 2004) near the end of the Pleistocene glaciation (~18,000 to 11,000 years ybp). These studies also generally equate expansion with an increase in the spatial extent of a species (i.e. dispersal into previously glaciated regions). A large scale spatial expansion of *Q. aurea* within the Guadalupe/San Antonio system is unlikely 40,000 years ago - Southeast Texas was never ice bound during the Pleistocene and the Guadalupe and San Antonio rivers were likely already connected at the time (Blum and Price 1998). On the other hand, the Pleistocene was a period of extreme variability in climate conditions (EPIC 2004) and the potential exists for the development of climate conditions optimal for *in-situ* population growth in *Q. aurea*.

In contrast, the timing of population contraction for the San Antonio is estimated to have begun approximately 2000 ybp in the late Holocene during the late Archaic period of human occupation of North America (Goebel et al. 2008), but the average of 10% quantile estimates are only 21 ybp. Determining the factors that contributed to the timing of population change is outside of the scope of this study, but the fact that the MSVAR posterior distribution

for timing of change in the San Antonio River overlaps with modern human occupation should be noted. The late timing of change in the San Antonio also means the population could have expanded with the Guadalupe/San Marcos in the late Pleistocene and then contracted later. Again, the analysis for the San Antonio was not as consistent as the Guadalupe/San Marcos due to poor model mixing and should not be consider as definitive. Future research should consider the addition of more microsatellite markers to strengthen the MSVAR analysis and reduce the level of uncertainty in population size and timing of change estimates.

While this study cannot identify the specific ecological factors that operated in the distant past to produced population change in these systems, the importance of the MSVAR results to present day conservation efforts lie in the indication that *Q. aurea* abundance differs between the lower Guadalupe/San Marcos and the lower San Antonio. Depending on the underlying reasons for this difference conservation strategies may need to be tailored to address different challenges to the persistence of *Q. aurea* and other mussel species in these systems. Further research should attempt to clarify if this contemporary difference in abundance is related to variation in ecological factors, such as the patch availability or fish host density and distribution, or if it can be attributed to variation in anthropogenic impacts between systems.

5.9 Final Conclusions

The results of this research indicate the lower region of the Guadalupe/San Marcos River system is an important stronghold for several Central Texas endemic freshwater mussel species. Three of the four endemic species that historically inhabited the Guadalupe/San

Antonio system and were petitioned for listing under the Endangered Species Act were discovered in the Guadalupe River. Surveys revealed the existence of two reproducing populations of *F. mitchelli*, a species once thought to be extinct, but they also suggest the loss of another species, *Q. petrina*, from its historic range in the San Antonio River. While survey efforts suggests *Q. aurea* may be more abundant than previously thought in some sections of the lower Guadalupe River, overall mussel abundance in the San Antonio may be limited by the availability of suitable habitat.

The maintenance of genetic diversity is becoming fundamental to the management of threatened and endangered species (Frankham 2010). Consequently, the decline in abundance and diversity of freshwater mussels makes the preservation of genetic diversity a conservation concern for many species. The results of this study are encouraging in that populations of *Quadrula aurea* still contain a significant amount of neutral genetic diversity and diversity is distributed among all the populations tested. Populations are essentially panmictic at moderately large spatial scales and high gene flow among populations likely reduces the potential for diversity loss due to drift. Moreover, our estimates for N_e compare favorably with the range of N_e values (500 – 5000) that have been recommended by various authors for the maintenance of a genetically viable population (Franklin 1980; Lande 1995; Reed et al. 2003; Traill et al. 2007). However, these results should be viewed with caution from a conservation perspective - relatively high levels of neutral genetic diversity have been reported for other freshwater mussels with well documented declines in range extent and abundance (Jones et al. 2015; Roe and Boyer 2015). The reasons for this discrepancy are not clear, but there is a time lag between the reduction in effective population size and the associated decline in genetic

diversity (Waples 2010). This coupled with the fact that freshwater mussels can have long lifespans means changes that took place as a result of fragmentation events (i.e. dam construction) in the early 1900's may not be fully manifest in the genetic structure of *Q. aurea*.

This work sets a baseline for monitoring change over time in *Q. aurea* and for evaluating the delayed effects of fragmentation. In addition, this work is relevant to two other threatened quadruleds in Texas (*Quadrula petrina* and *Quadrula houstonensis*) that likely utilize channel catfish as host species and may exhibit similar genetic structure.

CHAPTER 6

NOTE ON THE HABITAT CONDITIONS FOR *Fusconaia mitchelli**

Abstract—The false spike (*Fusconaia mitchelli*) is a freshwater bivalve native to Central Texas and thought to be extinct until a small population was located on the Guadalupe River in 2011. Although the newly discovered population documented the continued existence of the false spike, it consisted solely of mature adults and the reproductive status of the species was still in question. In the fall of 2012 we located a small population of the false spike in another section of the Guadalupe River that consisted of adults and juveniles and was clearly reproducing. The identification of a reproducing population of such a critically endangered and cryptic species offers a unique opportunity to document much needed habitat information. We present here the habitat conditions where this population was found in order to help inform the regulatory assessment of the species.

Resumen— El false spike (*Fusconaia mitchelli*) es un bivalvo de agua dulce nativa de Texas Central y pensó que se había extinguido hasta una pequeña población se encuentra en el Río Guadalupe en el año 2011. A pesar de que el recién descubierto población documentada la existencia continuada de la false spike, que consistía únicamente en los adultos maduros y el estado reproductivo de la especie estaba todavía en duda. En el otoño de 2012 se encuentra una pequeña población de la false spike en otra sección del Río Guadalupe que consistía de adultos y menores y fue claramente reproducir. La identificación de una población reproductora en grave peligro de extinción tales especies crípticas y ofrece una oportunidad

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única de documento muy necesaria información de hábitat. Presentamos aquí las condiciones del hábitat en que esta población fue encontrado con el fin de ayudar a informar sobre la evaluación de la normativa de la especie.

Native freshwater bivalves (families Margaritiferidae and Unionidae) comprise the most imperiled faunal group in North America. Of the approximately 300 species found in the United States 56% are believed to be at risk to some degree (i.e., classified as vulnerable, imperiled or critically imperiled) while another 13% are classified as either presumed or possibly extinct (Master et al., 2000). Texas is home to over 50 species of unionid bivalves (Howells et al., 1996) and currently has 15 species, seven of which are endemic, listed as threatened within the state. Six of Texas' threatened unionids were classified by the U.S. Fish and Wildlife as candidates for listing under the Endangered Species Act in 2011 (USFWS, 2011) and an additional six species are currently under status review (USFWS, 2009).

The false spike (*Fusconaia mitchelli*) is an endemic central Texas unionid bivalve that is currently under review for listing and was presumed extinct (Howells et al., 1996; Haag, 2009a; Howells, 2011) until a very-recently deceased individual was found on the San Saba River in 2011 (Randklev et al., 2011). Subsequent surveys produced 7 live specimens of *F. mitchelli* in one location on the Guadalupe River in south-central Texas (Randklev et al., 2011). These findings demonstrate the continued existence of *F. mitchelli* in central Texas, but it is important to note that all of the live specimens found were adults in the same size class. Freshwater mussels can be long-lived with life spans ranging from <10 to approximately 130 years (Haag, 2009b) and functionally extinct populations, composed solely of non-reproducing adults, have been reported from heavily altered river systems (Layzer et al., 1993; Hughes and Parmalee,

1999). The lack of a range of size classes in the only known extant population of *F. mitchelli* implied the reproductive status of the species was still questionable.

In the fall of 2012 we located eight live specimens of *F. mitchelli* during a survey on the Guadalupe River near Cuero, Texas. The recently dead shells of an additional six specimens were also found at the same location. Field identification of *F. mitchelli* was verified by Robert Howells - a 22 year veteran of the Texas Parks and Wildlife Department and a recognized expert on freshwater mussels in Texas - using the recently dead shells. Both live and dead specimens represented a range of size classes; shell lengths for the live specimens were 31.5 - 57.5 mm while lengths of the recently dead were 23.8 - 48.0 mm. Because shell length is an indicator of relative age in freshwater bivalves (Harmon and Joy, 1990, Haag, 2009b) this finding constitutes the first documentation of a reproducing population of *F. mitchelli* in at least 30 years (Howells, 2011).

Identifying a reproducing population of *F. mitchelli* is of critical importance because very little is known about the species' biology or habitat requirements (Howells et al., 1996; Howells, 2010) and information is needed to facilitate the U.S. Fish and Wildlife review process (U.S. FWS, 2009). A reproducing population implies the presence of ecological conditions sufficient for population persistence and represents a rare opportunity to further our knowledge of the habitat requirements of this species. To this end, we measured a set of physical variables commonly assessed in riverine studies (i.e., wetted channel width, channel slope, flow velocity, water depth, and substrate composition) to document the general habitat conditions where this population was found. We then used water velocity and depth measurements to estimate a set of complex hydraulic variables (Table 3.2) that have been shown to interact with substrate

composition and influence the suitability of mussel habitat (Hardison and Layzer, 2001; Gangloff and Feminella, 2007; Zigler et al., 2008; Allen and Vaughn, 2010). We used the particle size distribution from our sediment sample to develop an estimate of relative shear stress (RSS) to evaluate substrate stability under the flow conditions present at the time of sampling. RSS is defined as the ratio of the friction force acting on the substrate (shear stress) to the friction force required to set a given particle size in motion (critical shear stress) (Morales et al., 2006). Habitat patches that experience RSS values ≥1 during spates are considered unstable and represent poor quality mussel habitat (Morales et al., 2007; Allen and Vaughn, 2010).

We used a Marsh-McBirney™ Flo-Mate flowmeter (Marsh-McBirney, Frederick, Maryland) and a 1.5 m top setting wading rod to measure water velocity and depth at five equally spaced locations (0.1, 0.3, 0.5, 0.7, and 0.9× channel width) along a single transect bisecting the center of the mussel bed. At each transect point we measured water velocity at 60% of depth and again at 5 cm above the substrate to characterize near-bed velocity. We used a survey level and stadia rod to measure the change in water surface elevation (channel slope) over the length of the mussel bed (47.3 meters). Substrate composition was relatively uniform across the mussel bed; therefore, we collected a single sediment core (16.5 cm diameter x 8 cm deep) from the center of the mussel bed with a custom designed sampler constructed of PVC pipe and designed to work in course (gravel to cobble) substrate. The sediment sample was returned to the University of North Texas where it was oven dried at 110° F for 24 hours, ground with a mortar and pestle to separate aggregated particles, and sieved to partition substrate size fractions. Sieve size fractions were 32, 22.6, 16, 11, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.063 mm. After separation we weighted each size fragment to the nearest 10th of a gram.

Sediment particle size classes and complex hydraulic variables, described in Table 1, were calculated using formulas from Allen and Vaughn (2010) and references therein. We calculated values for each hydraulic variable at each of the 5 transect points using the near-bed velocity measurements. We took the arithmetic average of shear stress (τ) as an estimate of mean shear stress ($\bar{\tau}$) for the mussel bed. Mean shear stress was compared to the critical shear stress (τ_c) estimate for the median particle size (D_{50}) to develop an estimate of RSS for the entire mussel bed. We used a value of 0.07 for the Shield's parameter (θ_c) in the calculation of critical shear stress because the channel substrate was composed primarily of packed gravel and sand, but with a slight armoring of larger sized particles on the surface (Gordon et al., 2004).

The *Q. mitchelli* population we discovered is located in a shallow run just upstream of a moderately sized riffle. Adjacent land-use is characterized by pasture with an intact riparian zone of approximately 200 meters on the left bank and 20 meters on the right bank. Overall habitat conditions were homogeneous throughout the short reach and were characterized by relatively shallow depth, moderate flow velocity, and a packed sand and gravel substrate. The upper surface of the substrate was composed of larger particles (>3.2 cm) that appeared to stabilize the smaller particles beneath. Wetted channel width was 42 m and the longitudinal length of channel surveyed was 47 m equaling a total survey area of approximately 1974 m^2. The channel cross-section was trapezoidal with a mean water depth of 0.53 m (*SD* = 0.11 m). Channel slope over the length of the mussel bed was 0.00013 m/m, mean water velocity at 60% of depth was 0.79 m/s (range 0.62-0.97 m/s), and mean near-bed velocity was 0.45 m/s (range 0.36-0.55 m/s). Median particle size (D_{50}) was 1.1 cm, D_{16} was 0.025 cm, D_{84} was 3.2 cm, mean particle size ($\overline{D}$) was 1.34 cm, and bed roughness was calculated as 11.2 cm.

Estimates of complex hydraulic variables varied across our single transect; Fr ranged from 0.16-0.23 with a mean value of 0.20, Re_* ranged from 4055-5998 with a mean of 5076, and τ ranged from 14.06-28.63 dynes/cm^2 with a mean of 21.1 dynes/cm^2. We estimated a value of 124.8 dynes/cm^2 for the critical shear stress (τ_c) from our single sediment sample and a value of 0.17 for RSS. Theoretically, an RSS value ≥1 is needed to initiate substrate motion (Morales et al., 2006) and our estimate is well below the critical threshold for substrate movement. However, our RSS value and other complex hydraulic variables were calculated from measurements taken during low flow conditions and must be viewed in this context. Empirical evidence suggests that substrate stability under high flow conditions is a more relevant measure of habitat suitability for freshwater mussels (Allen and Vaughn, 2010) and thus complex hydraulic variables calculated during low flow conditions may be of limited value. Nevertheless, our intent with this note is to document the habitat conditions supporting this population of critically endangered *F. mitchelli* and the addition of low-flow associated information provides some insight into the velocity-substrate environment in which it was found. We hope this note will spur research efforts and contribute to the development of more clearly defined critical habitat requirements for this highly endangered species.

APPENDIX A

FORMULAS FOR EFFECT SIZE CALCULATION

To calculate the standardized mean difference between two groups, subtract the mean of one group from the other (M1 – M2) and divide the result by the pooled standard deviation (SD) for the population from which the groups were sampled.

$$Hedges'\ g = \frac{M_1 - M_2}{SD_{pooled}}$$

The recommended approach for calculating the pooled standard deviation if the groups are dissimilar in size is to weight each group's standard deviation by its sample size (*n*). To calculate the weighted and pooled standard deviation (SD_{pooled}) we use the following equation from Hedges (1981):

$$SD_{pooled} = \sqrt{\frac{(n_1 - 1)SD_1^2 + (n_s - 1)SD_2^2}{n_1 + n_2 - 2}}$$

Hedges' *g* was also developed to remove a small positive bias affecting the calculation of *d* (Hedges 1981). An unbiased version of *d* can be calculated using the following equation from Hedges and Olkin (1985):

$$g \cong d\left(1 - \frac{3}{4(n_1 + n_2 - 2) - 1}\right)$$

Calculation of approximate 95% confidence intervals uses the asymptotic standard error SE:

$$SE = \sqrt{\frac{n_1 + n_2}{n_1 n_2} + \frac{g^2}{2(n_1 + n_2 - 2)}}$$

The approximate width of 95% CIs for the effect (*g*) size is calculated as:

Upper CI = $g + 1.96 \times SE$

Lower CI = $g - 1.96 \times SE$

APPENDIX B

FULL SURVEY RESULTS

Table B1. Survey locations, search times, and numbers of three state threatened endemic freshwater mussels found by river.

Basin	Site Location	County	Latitude	Longitude	Date	Man Hours	*Quadrula aurea*	*Quadrula petrina*	*Fusconaia mitchelli*
Guad	Above Hwy 1117 near Segin, TX	Guadalupe	29.54027	97.88938	6/8/2014	3.5	32	0	0
Guad	3 km below Lake Wood Dam	Gonzales	29.47084	-97.4736	8/6/2012	1.5	58	0	0
Guad	Below Hwy 183 near Gonzales, TX	Gonzales	29.48787	97.43587	8/4/2013	0.5	12	4	0
Guad	Below Hwy 183 near Gonzales, TX	Gonzales	29.48105	97.43975	8/4/2013	0.5	6	5	1
Guad	Below Hwy 183 near Gonzales, TX*	Gonzales	29.49194	97.4314	8/4/2013	2	62	32	13
Guad	Above FM 776 near Cuero, TX*	Dewitt	29.16184	97.32478	9/21/2012	3.25	47	9	8
Guad	Below Hwy 77 near Victoria, TX*	Victoria	28.83119	97.05953	9/23/2012	1.5	53	10	1
Guad	Above confluence with San Antonio	Victoria	28.51565	96.89661	8/2/2013	0.33	0	0	0
Guad	Above confluence with San Antonio	Victoria	28.52385	96.89993	8/2/2013	0.33	0	0	0
Guad	Above confluence with San Antonio	Victoria	28.54046	96.90085	8/2/2013	0.33	0	0	0
Guad	Above confluence with San Antonio	Victoria	28.55195	96.89653	8/2/2013	0.33	0	0	0
Guad	Above confluence with San Antonio	Victoria	28.5715	96.91826	8/2/2013	0.33	0	0	0
Guad	Above confluence with San Antonio	Victoria	28.53689	96.90149	8/2/2013	0.33	0	0	0

Table B1 cont. Survey locations, search times, and numbers of three state threatened endemic freshwater mussels found by river.

Basin	Site Location	County	Latitude	Longitude	Date	Man Hours	*Quadrula aurea*	*Quadrula petrina*	*Fusconaia mitchelli*
San Antonio	Above confluence with Guadalupe	Victoria	28.50871	96.8979	8/3/2013	0.33	0	0	0
San Antonio	Above confluence with Guadalupe	Victoria	28.51332	96.90553	8/3/2013	0.33	0	0	0
San Antonio	Above Hwy 72 near Runge, TX	Karnes	28.85203	97.75233	6/25/2013	0.33	1	0	0
San Antonio	Above Hwy 72 near Runge, TX	Karnes	28.85302	97.73429	6/25/2013	0.33	2	0	0
San Antonio	Above Hwy 72 near Runge, TX	Karnes	28.84977	97.74905	6/25/2013	0.33	5	0	0
San Antonio	Above Hwy 72 near Runge, TX*	Karnes	28.85164	97.74628	6/25/2013	1	22	0	0
San Antonio	Above Hwy 236 near Charco, TX*	Goliad	28.74972	97.6515	6/27/2013	1	31	0	0
San Antonio	Below Hwy 183 near Goliad, TX*	Goliad	28.6504	97.38259	8/20/2012	1	59	0	0
San Antonio	Above Hwy 183 near Goilad	Goliad	28.65255	97.38941	8/1/2015	2	74	0	0
Cibolo Creek	Below FM 81 near Panna Maria, TX	Karnes	28.97184	97.87460	6/26/2013	0.75	4	0	0
San Marcos	Below Hwy 80 below Luling, TX	Guadalupe / Caldwell	29.65381	97.63168	8/4/2012	1.5	2	9	0
San Marcos	Below Hwy 80 below Luling, TX	Guadalupe / Caldwell	29.64013	97.62817	8/3/2012	1.5	6	10	0
San Marcos	At Palmetto State Park	Gonzales	29.58839	97.58569	9/6/2013	1	33	12	0
San Marcos	Below Hwy 90 above Luling, TX	Guadalupe / Caldwell	29.66742	97.68513	8/14/2013	1.5	40	51	0

APPENDIX C

HABITAT MEASUREMENTS

Table C1. Date, location, and general stream size measurements collected at eleven *Q. aurea* mussel beds. m, meter

Site	Date	Latitude	Longitude	Bed width (m)	Wetted width (m)	Bankfull width (m)
Runge	6/25/2013	28.851640	-97.746280	2.6	22.9	23.8
Charco	6/27/2013	28.749720	-97.651500	6.0	19	31
Goliad	8/20/2012	28.650314	-97.382713	10.0	16.5	19.6
Vacero	8/1/2015	28.652546	-97.389406	5.0	19.5	21
Mueller	6/9/2011	28.645390	-97.352830	3.0	24.20	27.4
Victoria	9/23/2012	28.831190	-97.059530	14.0	14	32
Cuero	9/22/2012	29.161840	-97.324780	42.0	42	44
Gonzales	8/4/2013	29.491940	-97.431400	34.0	34	35.4
Lake Wood	8/6/2012	29.470840	-97.473565	8.0	37.3	38.8
Segin	6/8/2014	29.540270	-97.889380	27.5	27.5	29.5
Palmetto	9/6/2013	29.588386	-97.585686	13.0	23.8	26.5
Luling	8/14/2013	29.667420	-97.685130	20.2	20.2	20.2

Table C2. Stream depth measurements (meter) collected at eleven *Q. aurea* mussel beds.

Site	Depth 1	Depth 2	Depth 3	Depth 4	Depth 5		Bankfull Depth 1	Bankfull Depth 2	Bankfull Depth 3	Bankfull Depth 4	Bankfull Depth 5
Runge	0.14	NA	0.9	NA	1.21		1.04	NA	1.8	NA	2.11
Charco	0.49	0.96	1.05	1.3	1.4		1.39	1.86	1.95	2.2	2.3
Goliad	0.15	0.28	0.39	0.54	0.63		0.7	0.88	0.98	1.08	1.14
Vacero	0.22	0.62	0.7	0.82	0.82		0.82	0.84	1.32	1.52	1.64
Mueller	0.38	NA	0.54	NA	0.55		0.82	NA	0.94	NA	1.02
Victoria	0.28	0.45	0.35	0.22	0.07		0.84	1.01	0.91	0.78	0.63
Cuero	0.34	0.68	0.58	0.5	0.57		1.24	1.58	1.48	1.4	1.47
Gonzales	0.32	0.45	0.54	0.4	0.31		0.72	0.85	0.94	0.8	0.71
Lake Wood	0.32	0.49	0.58	0.66	0.98		1.12	1.31	1.38	1.38	1.46
Segin	0.28	0.8	1	1	0.6		0.78	1.3	1.5	1.5	1.1
Palmetto	0.3	0.32	0.48	0.56	0.62		0.7	0.72	0.88	0.96	1.02
Luling	0.52	0.52	0.46	0.5	0.49		1.02	1.02	0.96	1	0.99

Table C3. Flow velocity measurements (ft/sec) collected at eleven *Q. aurea* mussel beds. %, percent; NB, nearbed

Site	60% Vel 1	60% Vel 2	60% Vel 3	60% Vel 4	60% Vel 5		NB Vel 1	NB Vel 2	NB Vel 3	NB Vel 4	NB Vel 5
Runge	0.08	NA	0.23	NA	0.22		0.08		0.05		0.08
Charco	0.35	0.93	0.85	0.73	0.81		0.24	0.18	0.25	0.5	0.45
Goliad	0.29	0.78	1.27	1.54	1.81		0.26	0.52	0.82	0.88	1.14
Vacero	0.18	0.52	0.85	1.37	1.84		0.15	0.11	0.35	0.66	1.15
Mueller	0.1	NA	0.41	NA	0.53		0.01	NA	0.17	NA	0.24
Victoria	2.02	2.95	2.55	1.47	NA		1.67	1.96	1.8	1.12	0.045
Cuero	2.03	3.18	2.69	2.14	2.98		1.35	1.32	1.81	1.18	1.8
Gonzales	2.2	2.1	1.98	1.75	1.23		1.52	1.5	1.55	1.4	1.12
Lake Wood	-0.05	0	-0.04	-0.1	0.01		-0.05	0	-0.06	-0.06	0.04

Segin	0.66	0.59	0.65	0.6	0.55		0.42	0.38	0.28	0.38	0.37
Palmetto	0.79	0.78	1.26	2.36	1.96		0.22	0.7	1.21	1.18	0.91
Luling	0.42	0.64	0.9	1.14	1.1		0.03	0.33	0.53	0.9	0.7

Table C4. Gradient measurements collected at eleven *Q. aurea* mussel beds. US, upstream; DS, downstream; in, inches; m, meters; k, kilometers.

Site	Height US (in)	Height DS (in)	Difference (in)	Difference (m)	Distance (m)	Gradient (m/m)	Gradient (m/k)
Runge	17.75	18.50	0.75	0.01905	36.2	0.000526243	0.526243
Charco	50.625	51.0	0.375	0.009525	42.0	0.000226786	0.226786
Goliad	34.80	35.0	0.20	0.00508	37.0	0.000137297	0.137297
Vacero	36.25	36.50	0.25	0.00635	35.0	0.000181429	0.181429
Mueller	35.0	35.25	0.25	0.00635	41	0.000154878	0.154878049
Victoria	37.50	40.50	3.0	0.0762	40.0	0.001905	1.905
Cuero	44.25	44.50	0.25	0.00635	47.3	0.000134249	0.134249
Gonzales	51.625	52.25	0.625	0.015875	48.0	0.000330729	0.330729
Lake Wood	36.40	36.60	0.20	0.00508	40.0	0.000127	0.127
Segin	50.75	51.25	0.50	0.0127	50.0	0.000254	0.254
Palmetto	36.00	37.0	1.0	0.0254	32.0	0.00079375	0.79375
Luling	40.50	41.0	0.5	0.0127	36.0	0.000352778	0.352778

Table C5. Weight (g) of sediment retained by sieve size for sediment samples collected at eleven *Q. aurea* mussel beds.

Site	<.063 mm	0.063 mm	0.125 mm	0.25 mm	0.5 mm	1 mm	2 mm	4 mm	8 mm	16 mm	32 mm	64 mm
Runge	28.6	40.5	37.2	10.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Charco	8.1	30.3	284.8	520.5	158.7	93.3	47.3	51.3	103.2	349.9	1289.7	0.0
Goliad	14.7	27.0	102.3	381.0	204.2	36.6	27.2	52.9	121.3	249.1	596.9	0.0
Vacero	41.9	32.3	89.3	345.2	115.1	35.9	35.0	87.6	226.3	590.3	894.0	0.0
Mueller	244.6	363.3	1435.3	1439.3	221.7	10.1	3.0	4.8	0	0	0	0
Victoria	4.0	5.0	90.4	271.6	132.0	83.0	182.9	234.7	299.6	450.9	515.1	894.6
Cuero	5.5	7.1	74.4	356.4	180.7	47.0	47.8	141.7	280.3	525.8	612.9	0.0
Gonzales	3.9	15.8	141.2	213.9	89.3	116.2	164.5	270.4	443.1	782.3	1325.8	0.0
Lake Wood	10.9	20.3	36.01	28.85	6.5	0.6	0.2	0.9	0	0	0.0	0.0
Segin	17.4	38.9	123.9	75.0	21.2	35.7	83.0	177.7	484.9	1196.3	1174.5	0.0
Palmetto	11.3	25.5	167.0	103.2	37.1	23.8	27.5	57.3	135.6	220.6	628.7	0.0
Luling	39.4	31.8	88.9	289.3	208.1	160.0	218.4	351.5	522.6	683	630.4	0.0

Table C6. Land cover (%) determined through GIS analysis and complex hydrologic variables (unit-less) calculated for eleven *Q. aurea* mussel beds. RSS, relative shear stress; Re, Reynolds number; Fr, Froud number.

Site	Developed	Forest	Agriculture	Shrubland	Herbaceous		RSS	Re	Fr
Runge	0.05	0.04	0.526	0.279	0.056		0.016	0.8	0.015
Charco	0.04	0.05	0.585	0.259	0.031		0.004	1610	0.031
Goliad	0.07	0.21	0.332	0.293	0.029		0.066	4166	0.111

Vacero	0.06	0.24	0.309	0.29	0.019		0.022	2977	0.074
Mueller	0.27	0.1	0.293	0.245	0.03		0.046	3.9	0.023
Victoria	0.07	0.12	0.569	0.117	0.028		0.252	26589	0.227
Cuero	0.03	0.16	0.407	0.269	0.023		0.157	7229	0.202
Gonzales	0.13	0.08	0.564	0.143	0.022		0.129	8583	0.219
Lake Wood	0.05	0.09	0.626	0.152	0.019		0.006	1.1	0.007
Segin	0.29	0.1	0.287	0.239	0.029		0.006	1910	0.046
Palmetto	0.1	0.29	0.278	0.245	0.019		0.043	5132	0.119
Luling	0.16	0.14	0.309	0.32	0.03		0.033	2016	0.069

Table C7. Percent soil composition by STATSGO2 category determined through GIS analysis for eleven *Q. aurea* mussel beds.

	Runge	Charco	Goliad	Vacero	Mueller	Victoria	Cuero	Gonzales	LakeWood	Segin	Palmetto	Luling
s7163	0	0	0	0	0	0	0	0	0	3.94	0	0
s7221	0	0	0	0	0	0	0	0	0	31.73	0	0.27
s7265	0	0	0	0	0	0	0	12.44	17.82	45.61	50.3	72.98
s7311	0	0	0	0	0	0	0	0	21.49	0	0	0
s7322	0	0	7.81	7.36	6.36	0	0	0	0	0	0	0
s7332	0	0	0	0	0	0	18.86	0	0	0	0	0
s7344	0	0	0	0	0	0	0.15	0	0	0	0	0
s7353	0	0	0	0	0	0	0	1.33	0	0	0	0
s7377	0	0	0	0	0	0	0	0	0	0	8.97	12.56
s7415	0	0	0	0	0	13.17	0	0	0	0	0	0
s7430	0	9.23	9.21	8.41	17.39	0	0	0	0	0	0	0
s7462	0	0	0	0	0	31.81	38.97	53.58	53.04	0	25.78	14.18
s7467	0	0	0	0	0	0	0	4.2	7.64	0	0	0
s7486	3.67	0	0	0	0	0	0	0	0	0	0	0
s7497	0	0	0	0	0	0	0	23.46	0	0	0	0
s7525	0	0	0	0	0	0	0	4.99	0	0	14.95	0
s7546	21.41	0	0	0	0	0	42.02	0	0	0	0	0
s7660	0	0	0	0	0	17.38	0	0	0	0	0	0
s7663	0	0	0	0	0	0	0	0	0	18.73	0	0
s7675	0	0	0	0	0	32.15	0	0	0	0	0	0
s7716	0	0	1.53	1.74	0	0	0	0	0	0	0	0
s7718	0	31.03	38.7	38.28	37.35	5.48	0	0	0	0	0	0
s7719	39.88	36.99	16.14	18.04	9.75	0	0	0	0	0	0	0
s9710	29.45	22.75	26.61	26.16	29.16	0	0	0	0	0	0	0
s9712	5.59	0	0	0	0	0	0	0	0	0	0	0

APPENDIX D

GENETIC DATA

Genetic data for individual *Quadrula aurea* specimens by population and locus. Numbers represent allele sizes in terms nucleotide length. Question marks (?) represent missing alleles due to non-amplification.

Population = Runge												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
SA_RU1	233	250	153	153	237	274	222	226	241	245	172	176
SA_RU2	240	244	153	153	237	250	230	230	241	245	165	189
SA_RU3	242	264	153	179	229	246	169	186	237	253	172	187
SA_RU4	244	246	153	153	233	237	174	218	241	249	156	165
SA_RU5	258	258	153	153	246	262	218	230	245	245	165	172
SA_RU6	242	246	153	153	233	250	178	202	241	257	156	189
SA_RU7	240	254	170	183	233	233	202	226	245	253	156	169
SA_RU8	250	269	153	153	242	246	226	238	225	245	172	181
SA_RU9	235	252	153	153	233	246	222	226	249	257	165	189
SA_RU10	233	233	153	153	233	237	202	226	229	241	156	174
SA_RU11	235	248	153	153	246	254	206	210	229	245	167	179
SA_RU12	233	244	153	153	229	274	210	230	233	253	165	189
SA_RU13	242	264	153	153	233	237	234	246	241	253	156	165
SA_RU14	242	244	153	153	233	250	210	242	249	253	156	156
SA_RU15	233	256	153	153	237	246	182	222	241	249	187	187
SA_RU16	250	267	153	170	233	233	214	222	225	253	172	176
SA_RU17	246	254	153	153	233	262	206	230	241	253	167	181
SA_RU18	248	250	153	153	233	233	226	242	241	253	187	191
SA_RU19	254	254	153	179	233	233	210	234	225	241	156	176
SA_RU20	248	252	153	153	233	237	190	214	237	245	189	189
SA_RU21	250	258	153	153	237	246	194	202	241	249	187	198
SA_RU22	235	262	153	170	237	237	202	226	237	245	167	169
Population = Charco												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
SA_Ch1	237	254	153	183	233	237	186	190	241	241	156	183
SA_Ch2	242	246	153	153	229	250	198	230	237	245	169	172
SA_Ch3	246	260	153	179	229	233	202	210	237	257	174	198
SA_Ch4	244	250	153	153	242	258	214	238	241	253	167	172
SA_Ch5	254	256	153	153	233	246	222	234	241	245	156	189
SA_Ch6	235	244	153	153	233	237	222	230	245	245	172	176
SA_Ch7	240	258	153	153	233	233	202	222	237	245	167	185
SA_Ch8	233	235	153	153	229	237	222	246	237	241	156	162
SA_Ch9	233	233	153	153	233	258	214	226	241	249	165	185
SA_Ch10	240	246	153	153	237	254	226	234	241	245	165	181
SA_Ch11	242	252	153	179	233	242	226	234	237	249	156	176
SA_Ch12	235	242	153	153	233	233	198	202	241	253	183	187
SA_Ch13	242	273	153	153	229	233	218	226	241	245	183	189
SA_Ch14	235	244	153	153	233	242	218	222	241	245	156	156

SA_Ch15	248	250	153	162	233	286	202	206	237	237	165	187
SA_Ch16	262	262	153	153	229	237	214	222	233	241	179	191
SA_Ch17	235	250	153	170	233	233	210	218	233	253	165	189
SA_Ch18	233	244	153	153	242	258	214	242	237	245	165	191
SA_Ch19	233	254	153	166	237	262	220	240	237	237	169	169
SA_Ch20	237	262	153	153	237	246	202	234	233	253	167	194
SA_Ch21	244	252	153	153	229	233	222	222	225	245	156	165
SA_Ch22	244	254	153	153	237	258	210	234	225	237	187	191
SA_Ch23	235	240	153	153	250	258	210	218	245	253	156	172
SA_Ch24	235	262	153	170	229	237	218	218	241	241	156	172
SA_Ch25	242	254	153	166	237	242	210	214	245	245	169	196
SA_Ch26	250	254	153	153	237	266	206	226	225	245	198	198
SA_Ch27	242	244	153	153	242	254	206	234	249	253	156	167
Population = Goliad												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
SA_G1	235	235	175	179	242	250	210	214	245	257	156	169
SA_G2	231	248	153	153	233	246	190	198	241	245	165	172
SA_G3	240	244	153	153	229	242	222	230	237	245	165	183
SA_G4	233	252	153	166	233	250	226	230	233	245	165	167
SA_G5	250	258	153	162	233	254	218	222	233	245	172	176
SA_G6	242	246	153	166	233	242	218	218	237	241	165	167
SA_G7	244	262	153	153	233	233	214	214	241	253	165	167
SA_G8	235	258	153	153	242	266	206	230	245	253	156	165
SA_G9	233	235	153	153	233	250	222	230	233	241	172	196
SA_G10	235	246	153	162	258	262	218	230	241	241	167	176
SA_G11	235	267	153	179	237	246	202	226	241	249	156	172
SA_G12	233	235	153	153	229	233	226	246	245	245	156	165
SA_G13	246	246	153	153	233	250	218	242	245	245	167	172
SA_G14	235	254	153	153	233	258	202	222	237	249	172	172
SA_G15	235	244	153	153	233	237	190	206	241	249	165	183
SA_G16	235	254	153	153	233	242	210	214	253	257	165	172
SA_G17	233	235	153	166	233	237	194	194	237	241	167	187
SA_G18	235	244	153	166	237	250	222	222	241	241	172	174
SA_G19	233	235	153	153	233	262	210	222	225	233	156	172
SA_G20	242	250	153	153	233	242	206	210	237	245	169	183
SA_G21	244	254	153	166	233	233	210	210	241	241	156	156
SA_G22	233	252	153	153	242	246	198	226	229	233	169	183
SA_G23	233	242	153	166	246	266	218	226	229	237	169	169
SA_G24	244	244	153	153	233	250	190	202	229	241	179	183
SA_G25	233	275	153	153	229	242	194	238	241	249	156	156
SA_G26	233	235	153	162	237	250	226	226	233	237	172	196
SA_G27	233	242	153	153	233	233	214	222	233	237	165	165
SA_G28	237	248	153	153	229	258	202	210	241	241	172	185

SA_G29	240	256	153	162	237	254	210	222	245	249	165	187
SA_G30	240	246	153	153	229	233	226	262	233	245	172	187
SA_G31	235	246	153	179	233	246	222	226	233	245	160	160
SA_G32	235	235	166	175	229	233	169	202	237	241	167	172
SA_G33	235	267	153	187	233	242	206	214	237	241	165	165
SA_G34	233	248	153	179	242	246	218	262	233	237	156	156
Population = Victoria												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
GR_UV1	233	256	153	162	233	237	178	210	237	257	167	169
GR_UV2	233	260	153	162	237	270	194	214	245	245	156	169
GR_UV3	246	252	153	153	237	237	190	202	237	245	174	174
GR_UV4	244	246	153	175	246	258	190	198	241	245	156	172
GR_UV5	235	246	153	162	233	254	165	210	237	241	156	160
GR_UV6	246	254	153	187	246	254	206	210	233	249	162	169
GR_UV7	248	252	153	179	237	237	202	238	241	245	156	176
GR_UV8	237	242	153	162	254	262	202	218	245	245	162	167
GR_UV9	246	248	153	153	237	250	190	210	237	241	156	156
GR_UV10	235	242	153	153	237	242	165	218	241	253	156	172
GR_UV11	231	235	153	166	229	254	178	238	237	237	156	167
GR_UV12	252	258	153	153	229	246	210	226	233	245	167	172
GR_UV13	240	244	153	162	233	246	214	218	241	245	167	176
GR_UV14	246	250	153	191	229	246	169	218	245	245	169	185
GR_UV15	240	244	153	153	246	250	198	214	229	241	167	169
GR_UV16	235	242	153	170	233	242	182	194	245	249	156	165
GR_UV17	242	244	153	153	237	250	190	242	241	249	160	162
GR_UV18	242	256	153	153	233	237	190	218	229	241	165	181
GR_UV19	244	256	153	162	233	258	190	218	241	249	156	156
GR_UV20	242	246	153	153	237	254	153	186	237	253	167	179
GR_UV21	244	250	153	153	233	242	210	226	237	245	156	165
GR_UV22	240	242	153	153	250	258	194	226	237	241	169	185
GR_UV23	242	244	153	153	229	233	190	206	225	237	156	176
GR_UV24	246	250	179	191	237	246	178	194	237	249	156	160
GR_UV25	240	246	153	162	225	237	206	218	249	249	165	165
GR_UV26	240	246	153	162	242	250	202	214	233	241	156	172
GR_UV27	240	250	153	170	233	242	194	202	237	237	156	169
GR_UV28	240	246	153	179	237	254	210	218	233	245	167	189
GR_UV29	235	240	153	166	237	242	198	198	229	241	167	176
GR_UV30	244	262	153	153	242	250	165	190	237	237	156	165
GR_UV31	244	254	153	153	242	250	186	194	245	257	165	165
Population = Cuero												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
GR_UC1	233	242	153	162	242	242	198	202	237	241	156	167
GR_UC2	237	244	162	175	233	237	218	234	237	249	176	176

GR_UC3	231	237	153	183	242	250	198	206	237	241	156	172
GR_UC4	244	244	153	153	233	250	153	202	237	249	165	167
GR_UC5	242	244	153	153	229	237	194	210	233	241	176	183
GR_UC6	242	246	153	153	233	233	174	242	233	237	167	169
GR_UC7	244	252	153	175	233	242	190	222	241	245	156	176
GR_UC8	240	256	153	153	237	242	153	210	245	245	156	176
GR_UC9	246	254	153	153	233	237	194	206	233	249	156	167
GR_UC10	242	254	153	162	229	233	206	222	245	245	167	169
GR_UC11	235	235	153	153	229	237	190	206	241	241	162	162
GR_UC12	242	258	153	175	242	258	202	214	241	249	160	194
GR_UC13	242	250	153	153	237	237	198	214	229	241	162	174
GR_UC14	244	254	153	153	233	237	218	218	237	237	165	174
GR_UC15	244	244	153	166	233	237	190	202	241	245	169	185
GR_UC16	246	246	153	153	242	246	190	202	241	249	156	167
GR_UC17	246	248	153	162	246	294	202	222	241	241	167	183
GR_UC18	246	260	162	162	246	250	157	226	241	241	156	156
GR_UC19	235	254	153	153	233	237	182	194	241	241	167	172
GR_UC20	242	248	153	153	250	266	174	206	237	241	160	176
GR_UC21	248	250	153	153	233	242	186	202	241	249	169	169
GR_UC22	240	246	153	166	229	246	206	214	237	245	176	179
GR_UC23	248	258	153	153	246	250	169	246	241	241	156	160
GR_UC24	248	256	153	153	242	246	182	194	237	241	158	174
GR_UC25	244	246	157	166	242	250	178	214	241	245	156	167
GR_UC26	240	244	153	153	246	246	165	238	241	241	165	174
GR_UC27	246	250	153	153	233	250	194	198	237	253	167	167
GR_UC28	242	250	153	153	233	242	218	222	237	253	156	172
Population = Gonzalez												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
GR_LZ1	246	248	153	153	246	254	157	202	249	253	181	189
GR_LZ2	240	246	153	175	233	233	218	226	241	249	167	179
GR_LZ3	235	252	153	166	242	246	198	218	249	249	156	169
GR_LZ4	242	248	153	166	229	258	214	218	241	245	156	176
GR_LZ5	246	252	153	153	233	242	190	194	241	245	172	179
GR_LZ6	235	256	153	175	233	266	165	218	229	237	167	172
GR_LZ7	240	258	153	153	246	254	210	218	241	245	156	167
GR_LZ8	235	240	153	170	237	254	198	206	237	241	167	169
GR_LZ9	231	240	153	153	242	258	186	210	241	245	158	169
GR_LZ10	242	246	153	153	237	242	194	218	241	257	167	174
GR_LZ11	240	258	153	162	254	266	198	234	233	241	165	169
GR_LZ12	242	248	153	153	237	242	153	202	237	241	162	167
GR_LZ13	235	250	162	162	233	242	165	206	241	245	160	167
GR_LZ14	233	258	153	166	233	250	210	218	237	261	167	169
GR_LZ15	246	246	153	153	246	250	210	214	241	245	156	165

GR_LZ16	237	240	153	166	229	233	165	206	233	237	156	160
GR_LZ17	240	246	175	183	237	250	178	202	241	249	156	169
GR_LZ18	244	252	153	175	233	237	206	214	241	249	167	179
GR_LZ19	242	244	153	153	242	258	190	222	237	253	172	181
GR_LZ20	237	242	153	162	229	229	182	206	245	245	172	172
GR_LZ21	252	267	153	153	237	237	206	226	237	237	156	183
GR_LZ22	242	244	153	166	233	242	206	218	237	245	156	162
GR_LZ23	240	267	153	153	250	254	222	234	237	249	169	179
GR_LZ24	237	242	157	170	237	242	206	230	237	241	156	176
GR_LZ25	240	246	153	196	237	242	169	194	237	241	162	167
GR_LZ26	246	258	153	162	233	242	210	222	241	245	156	183
GR_LZ27	240	250	153	166	237	246	206	218	245	249	156	165
GR_LZ28	233	254	153	175	262	266	190	218	229	245	156	169
GR_LZ29	246	246	153	179	242	258	169	186	237	253	156	169
Population = Lake Wood												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
GR_LW1	237	242	153	183	237	242	186	194	237	241	156	169
GR_LW2	242	250	153	153	237	254	198	210	237	257	156	167
GR_LW3	237	258	153	170	246	246	182	202	241	245	156	169
GR_LW4	235	244	153	162	258	278	153	202	225	245	156	176
GR_LW5	242	242	153	153	237	237	182	186	241	241	156	179
GR_LW6	242	244	153	153	250	254	198	218	241	249	156	174
GR_LW7	231	242	153	187	237	242	194	206	237	249	167	169
GR_LW8	235	242	162	196	237	258	202	238	237	253	156	156
GR_LW9	235	256	166	187	237	237	222	242	237	249	165	172
GR_LW10	242	246	153	179	242	262	210	222	229	245	156	167
GR_LW11	242	244	153	175	237	246	169	198	241	241	156	156
GR_LW12	240	262	162	162	242	246	198	210	237	249	160	165
GR_LW13	242	256	153	153	233	246	214	214	257	261	160	167
GR_LW14	242	248	153	153	237	237	206	230	249	261	169	169
GR_LW15	242	258	153	166	242	254	169	210	237	237	156	156
GR_LW16	231	258	153	153	250	262	206	230	249	269	169	169
GR_LW17	237	242	153	153	250	250	161	178	233	237	156	183
GR_LW18	242	260	145	179	242	250	169	210	237	241	156	176
GR_LW19	242	244	153	153	229	258	214	218	237	245	176	176
GR_LW20	231	242	153	153	233	233	202	210	233	245	156	179
GR_LW21	240	242	166	170	237	246	218	242	233	233	156	172
GR_LW22	242	264	166	166	229	250	190	214	237	241	167	172
GR_LW23	237	250	153	162	237	250	194	210	237	253	156	176
GR_LW24	244	248	153	162	233	278	198	214	237	241	167	176
GR_LW25	244	244	153	153	246	250	180	194	233	237	169	187
GR_LW26	244	250	153	153	237	254	210	250	241	245	169	169
GR_LW27	244	244	153	162	233	262	169	198	237	241	169	181

GR_LW28	242	246	153	153	229	262	174	218	249	253	169	185
GR_LW29	246	246	153	153	233	246	174	226	237	241	156	176
GR_LW30	235	242	153	153	237	262	174	210	237	257	167	172
Population = Palmetto												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
SM_PM1	231	246	153	170	242	274	214	230	233	241	156	169
SM_PM2	250	258	153	162	233	254	165	218	241	245	167	167
SM_PM3	254	267	162	170	237	274	182	246	237	241	156	172
SM_PM4	244	244	153	153	242	254	186	214	237	237	156	172
SM_PM5	240	240	153	166	233	258	169	182	233	237	156	167
SM_PM6	242	244	153	153	250	262	190	202	237	241	167	189
SM_PM7	235	248	153	153	242	246	169	210	237	241	169	169
SM_PM8	242	246	153	153	237	242	161	222	233	237	172	172
SM_PM9	242	244	153	153	229	250	198	234	233	245	156	167
SM_PM10	237	244	153	153	242	250	182	210	241	241	156	169
SM_PM11	242	246	153	162	250	254	210	226	229	233	160	165
SM_PM12	244	248	153	157	242	242	?	?	237	241	167	172
SM_PM13	244	250	153	153	237	250	178	186	245	253	167	167
SM_PM14	242	246	153	153	237	242	174	230	241	241	165	183
SM_PM15	250	256	153	153	237	250	198	206	237	249	165	169
SM_PM16	233	244	162	196	237	246	198	206	241	241	156	179
SM_PM17	235	237	153	170	237	254	153	222	241	245	156	156
SM_PM18	242	250	153	153	233	237	206	210	233	241	156	156
SM_PM19	240	246	153	166	233	246	198	206	237	245	174	191
SM_PM20	240	242	153	153	233	242	218	222	237	241	179	183
SM_PM21	244	246	162	170	237	237	210	214	233	241	156	169
SM_PM22	244	246	153	183	237	242	186	230	245	249	156	167
SM_PM23	235	242	153	179	233	237	198	198	233	237	156	156
SM_PM24	240	244	166	166	229	229	169	194	237	245	169	176
SM_PM25	240	242	153	175	233	250	202	226	225	245	156	167
SM_PM26	242	242	153	153	242	254	190	222	233	249	156	198
SM_PM27	237	242	153	153	250	254	202	210	245	249	174	176
Population = Luling												
	QfA130		**QfC109**		**QfD103**		**QfC6**		**QfC114**		**QfD5**	
SM_UL1	242	250	153	153	242	254	186	210	245	253	167	169
SM_UL2	240	240	153	166	237	250	198	210	249	253	165	169
SM_UL3	248	252	153	183	242	250	161	214	241	245	176	176
SM_UL4	237	258	153	166	233	250	198	214	233	241	167	169
SM_UL5	242	242	153	153	233	274	202	222	237	249	176	176
SM_UL6	244	250	153	170	250	274	226	230	237	253	156	156
SM_UL7	242	244	153	153	237	237	214	254	241	245	156	169
SM_UL8	237	240	157	162	242	242	169	194	253	257	167	169
SM_UL9	244	248	153	153	237	246	194	214	241	245	156	165

SM_UL10	252	258	153	153	254	266	214	230	237	237	172	179
SM_UL11	231	248	157	162	242	250	161	206	241	245	167	176
SM_UL12	242	252	153	153	242	242	178	198	237	245	158	165
SM_UL13	244	250	153	162	237	250	210	226	237	245	172	183
SM_UL14	244	267	153	170	237	237	169	202	?	?	174	174
SM_UL15	237	248	153	153	229	258	157	198	237	237	156	169
SM_UL16	242	248	153	153	237	250	182	198	233	253	167	172
SM_UL17	242	254	166	166	237	242	198	210	237	237	169	185
SM_UL18	242	244	153	153	250	254	194	194	241	245	160	169
SM_UL19	244	246	153	162	246	250	198	206	241	245	156	172
SM_UL20	235	240	153	175	233	254	194	218	241	249	156	176
SM_UL21	242	242	162	179	229	237	218	218	245	249	167	181
SM_UL22	242	254	153	153	233	242	194	206	241	241	169	183
SM_UL23	240	242	157	166	242	242	218	226	237	249	165	172
SM_UL24	233	256	153	162	233	233	214	218	229	237	158	167
SM_UL25	233	250	162	162	233	242	157	206	237	237	160	165
SM_UL26	246	250	153	153	229	233	182	202	245	245	156	156
SM_UL27	231	242	153	153	237	242	198	206	237	245	160	174
SM_UL28	231	256	153	153	237	237	194	206	233	245	167	174
SM_UL29	235	235	153	162	?	?	?	?	245	249	156	169
SM_UL30	254	262	153	153	237	237	198	206	245	253	160	172

APPENDIX E

SEQUENCING EXAMPLES

Nucleotide alignment of QfD5 sequences for seven *Q. aurea* individuals representing two alleles (6 of 156 bps and 1 of 174 bps) in comparison to one sequence from the source species *Q. fragosa*. The top line represents nucleotide position relative to the *Q. fragosa* sequence, blanks signify no difference between the source species and the *Q. aurea* sequences, and dashes (-) represent missing nucleotides in regions where microsatellite repeats vary among alleles or a result of the insertion/deletion of single nucleotides. The 6 *Q. aurea* sequences of 156 bps are numbered 1-6 while the single 174 bp sequence is numbered as 7.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
F	T	A	T	T	G	G	A	T	A	T	G	G	T	G	C	A	A	C	T	C	G	G	A	C	A	T	A	C	A	C	A	T	C	T	A	T	C	T	A	T	C	T	A	T	C	T	A	T	C	T	A	T	C	T	A	T	C	T	A	T
1																										C																																						
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4																										C																																					
5																										C																																					
6																										C																																					
7																										C																	A																						

P	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
F	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	G	T	G	A	T	T	T	A	T	T	T	T	A	A	T	C	A	T
1	G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									
2	G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									
3	G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									
4	G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									
5	G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									
6	G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									
7	G		G		G		G		G		G		G		G		G		G		G		G		G		G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C				-				-	-									

P	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169
F	G	T	A	T	G	T	C	T	G	T	A	A	G	A	A	A	A	T	G	T	T	A	C	T	C	A	G	T	G	C	T	C	A	A	G	T	G	T	G	A	C	T	A	T	G	G	T	T	G
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